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5 : 6-DITHIADECAMETHYLENE 1 : 10-BIS(TRIMETHYL-AMMONIUM IODIDE): A NEW MYONEURAL BLOCKING AGENT OF THE DEPOLARIZING TYPE

BY

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(RECEIVED MAY 27, 1952)

The compound 5:6-dithiadecamethylene 1:10-bis(trimethylammonium iodide) was synthesized by Andrews, Bergel, and Morrison (1953), and given the serial number Ro 3-0386. On preliminary investigation it proved to be a myoneural blocking agent with an action rather like that of decamethonium (Randall, personal communication). Since this latter drug has been found in clinical anaesthesia to have certain disadvantages (Gray, 1950 ; Hunter, 1950), it seemed worth while to study the new compound in some detail in the hope that it might be more satisfactory.

METHODS

In cats anaesthesia was induced by 40 mg./kg. of pentobarbitone given intraperitoneally. Maintenance doses of 5 to 10 mg./kg. were injected intravenously when the reflex contraction of the eyelids elicited by touching the inner canthus returned. In the curarized animal either an abnormally high blood pressure, over 185 mm. of mercury, not due to asphyxia, or irregular respiration was used as an indication for the administration of more pentobarbitone. In dogs, apart from the studies of ganglion blocking activity, only short experiments were carried out, and ether anaesthesia was used.

No direct records of muscular contraction were made. Instead the contraction of a foreleg muscle group in the cat was observed. Complete myoneural block was assumed to be present when stimulation of the appropriate fasciculus of the sciatic nerve, with what had previously been supramaximal shocks from an induction coil, no longer produced any visible movement of the unloaded foot or toes. Respiratory movements were recorded with the aid of tambours on the thorax and epigastrium. The mean blood pressure was measured with a mercury manometer with citrate as an anticoagulant.

Rabbit Head Drop Tests.—These were carried out on untrained animals. Small incremental doses of the drug were injected into the ear vein every 30 seconds until paralysis appeared. The dosage was so arranged that three to five increments were required to produce head drop.

Antagonists.—As antagonists are used in man to restore normal breathing, their action on the respiratory movements of experimental animals was used as the criterion of their effectiveness. In cats the relaxant was given until respiratory failure was indicated by depression of breathing movements and asphyxial hypertension. If the antagonist would then produce prompt restoration of respiration and reversal of the asphyxia, it was regarded as effective. These agents were also tested in less severe states of respiratory depression. They were given to animals in whom the relaxant drug had produced abolition of thoracic breathing and some reduction in the force of abdominal breathing. If both components of respiration were immediately restored the antidote was considered to be effective.

Antidotes were also studied in mice. A dose of the myoneural blocking agent was chosen which would kill a large percentage of the animals. The antidote was given, and after a suitable interval the selected dose of the myoneural blocking agent was given. Protection was assumed to have occurred if there was a significant reduction in the mortality in the second group.

Ganglion Blocking Activity.—This was tested directly for the parasympathetic only. Dogs and cats under pentobarbitone anaesthesia were submitted to bilateral vagotomy. The peripheral cut end of one vagus nerve was stimulated with an induction coil. The strength of the stimulus was the minimum which would produce a maximal response when applied to the adjacent sternomastoid muscle. Stimulation was continued until the blood pressure ceased to fall further, usually for some 10 to 15 seconds. After an interval of at least 5 minutes the drug to be tested was given,

and if it impaired the breathing artificial respiration was begun. If the same stimulus produced a fall in blood pressure of the same severity and duration it was considered that conduction in the parasympathetic ganglia was unimpaired. This method had previously been shown to be reliable when it was used to demonstrate the blocking effect of gallamine triethiodide on the terminal synapses of the cardiac vagus.

Results

The degree of myoneural block produced by various doses of Ro 3-0386 is given in Table I. For comparison the amounts of decamethonium with the same action are also given where the figures are available. The duration of the effect of comparable doses seemed to be about the same. The dose of Ro 3-0386 which would produce complete myoneural block in a nerve muscle preparation was just slightly less than that which would cause respiratory failure. At lighter levels of the drug's action, weakening of the response to single stimuli was apparent. Tetani were, however, fairly well sustained, a contrast to the effect of *d*-tubocurarine.

TABLE I
COMPARATIVE DOSES OF Ro 3-0386 AND DECAMETHONIUM

Species	Criterion of Myoneural Block	Myoneural Blocking Dose	
		Ro 3-0386 mg./kg.	Decamethonium mg./kg.
Mouse ..	LD50	1·95*	0·84†
Rabbit ..	Head drop	About 0·1	0·08–0·22
Cat ..	Paralysis thoracic breathing	0·01	—
	Apnoea	0·02	0·08
Dog ..	,,	About 0·1	—

* Parkes (personal communication). † Paton and Zaimis (1949).

Ro 3-0386 proved to have a muscle-stimulating action. Violent contractions of the limbs, enough to make the animal jump several inches into the air, quite often followed its intravenous administration in mice. Fasciculation and sometimes writhing movements could be observed when the drug was given to cats under pentobarbitone. These changes did not, however, appear in dogs anaesthetized with ether or pentobarbitone. When Ro 3-0386 was given by close intra-arterial injection into the tibialis anticus of the cat a sharp contraction of the muscle occurred, while when it was given into the femoral artery a less forceful response of all the muscles of the limb was seen. All these phenomena have been observed after the administration of decamethonium.

Action on Respiratory Movements.—In cats under pentobarbitone Ro 3-0386, like decamethonium, produced first intercostal paralysis with compensatory increase in the diaphragmatic movement. Then as the action of the drug became more profound diaphragmatic respiration became weaker and was lost in the fasciculation of the abdominal muscles which was a prelude to their complete paralysis. In three dogs under ether anaesthesia to which the drug was given in large doses the respiration was first depressed, and then, as the dose approximated to that which was going to kill the animal, there appeared jerky, gasping slow breathing which caused only very inadequate ventilation ; but this was sufficient to maintain the animal in a state of chronic suboxygenation until the paralysing effect of the drug had passed off. Similar breathing has been observed in human subjects who have been kept deeply under the influence of decamethonium for long periods (Gray, 1950 ; Hunter, 1950).

Summation with Decamethonium.—The results of the investigation so far indicated that Ro 3-0386 was a depolarizing blocking agent like decamethonium. It was therefore thought worth while to see whether a small dose of one drug would sum with a comparatively ineffective dose of the other. It was found that, if to a cat which received half the dose of Ro 3-0386 which would cause apnoea a similar half dose of decamethonium was given, breathing was stopped for a few minutes and a period of intercostal paralysis followed. A similar summation of action could be seen in mice, in which harmless doses of drugs given in quick succession caused death in a proportion of animals (Table II).

TABLE II
THE ADDITIVE EFFECTS OF DECAMETHONIUM AND Ro 3-0386

Drugs	Dose mg./kg. (about ½ LD50)	Number of Mice Died
Ro 3-0386	0·75	0/6
Decamethonium	0·4	0/2
Decamethonium and Ro 3-0386	{0·4 / 0·75}	5/6

Antagonists.—Hexamethonium given by intraperitoneal injection in doses of 40 mg./kg. failed to afford appreciable protection to mice against the muscle-paralysing action of Ro 3-0386 in doses of 1.0 mg./kg. In the cat hexamethonium (2 mg./kg. intravenously), a dose which in a previous study (Hunter, 1950) had produced reversal of the action of decamethonium, failed to cause any appreciable restoration of the breathing of animals partly

paralysed by Ro 3-0386. 3-Thiapentamethylene 1:5-bis(trimethylammonium iodide) (Ro 3-0438), however, in a dose of 2.0 mg./kg. produced a restoration of abdominal but not of thoracic respiration. On the other hand, neither Ro 3-0438 in a dose of 100 mg./kg. nor the related Ro 3-0468 (3-thiahexamethylene 1:6-bis(trimethylammonium iodide)) in doses of 50 mg./kg. by intraperitoneal injection reduced the mortality in mice subsequently given Ro 3-0386 in lethal doses.

Compound 49-204 of de Beer *et al.* (1951) (1-methyl - 2-(dimethylaminophenethyl) - piperidine-1:4′-bis-methiodide) in doses of 1.0 mg./kg. reversed the action of Ro 3-0386 in the cat, restoring both the diaphragmatic and thoracic components of breathing. After such a reversal the animals had an increased resistance to the myoneural blocking agent. In mice significant protection against lethal doses of Ro 3-0386 was given by 3.0 mg./kg. of compound 49-204 given intravenously five minutes before (Table III). This

TABLE III

THE PROTECTIVE EFFECT OF COMPOUND 49-204 AGAINST LETHAL DOSES OF Ro 3-0386

Drug	Dose mg./kg.	Mice Survived
Ro 3-0386	1·8	1/12
49-204	3·0	6/6
49-204 and Ro 3-0386 ..	{3·0 / 1·8}	9/12

$\chi^2=8{\cdot}4$. $P<0{\cdot}01$.

amount of the latter compound seemed unlikely to be responsible for any of the deaths, as it caused no deaths when given alone and is less than half the LD50 (7.2 mg./kg.; de Beer, personal communication).

Ro 3-0386 is also antagonized by gallamine triethiodide. Both in the cat and the dog the dose of the former agent needed to produce apnoea is increased in animals which have recovered from the muscle-paralysing action of the latter (Table IV). Mice which have received a sublethal dose of gallamine triethiodide (2 mg./kg.) are also protected to a significant extent against poisonous

TABLE IV

THE PROTECTIVE EFFECT OF GALLAMINE TRIETHIODIDE AGAINST THE ACTION OF Ro 3-0386 IN CATS AND DOGS

Animal	Myoneural Blocking Dose of Ro 3-0386 (mg./kg.)	
	Alone	After Gallamine Triethiodide
Cat	0·02 (3)	0·16 (2)
Dog	0·09 (4)	0·13 (2)

The figures indicate the means of a number of experiments (in brackets).

TABLE V

THE PROTECTIVE EFFECT OF GALLAMINE TRIETHIODIDE AGAINST THE ACTION OF Ro 3-0386

	Group	Dose of Ro 3-0386 mg./kg.	Mice Survived
Without gallamine triethiodide	I	1·2	0/6
	II	1·8	3/9
After gallamine triethiodide (2 mg./kg.)	I	1·2	4/6
	II	1·8	8/9

$\chi^2=7{\cdot}115$. $P>0{\cdot}02$, $<0{\cdot}05$.

doses of Ro 3-0386 (Table V). A more positive antagonism between the two drugs was seen when a temporary deepening in the respiration of a cat partially curarized by gallamine triethiodide was produced by the administration of 0.02 mg./kg. of Ro 3-0386.

Ganglion Blocking Activity.—Ro 3-0386 had no immediate effect on the blood pressure of any of the animals to which it was given. An asphyxial rise in blood pressure was observed when respiration failed, and it therefore seemed as if it did not block the sympathetic ganglia. This was confirmed directly when Pellmont (personal communication) found that Ro 3-0386 in doses of 0.03 mg./kg. had no effect on the conduction of impulses through the superior cervical ganglion of the cat. It did reduce the force of the contraction of the nictitating membrane, but the site of the block was distal to the superior cervical ganglion, since the response to postganglionic stimulation was equally reduced.

In the present study it was found that Ro 3-0386 had no effect on conduction through one type of parasympathetic ganglion. Doses up to twice that which would cause apnoea had no influence on the fall in blood pressure produced by stimulating the peripheral cut end of the vagus nerve.

The Use of Ro 3-0386 in Man

The methods used were similar to those employed in a previous study of decamethonium, i.e., the administration of a drug to patients already intubated and stabilized under plane I nitrous oxide and oxygen anaesthesia (Hunter, 1950). A barbiturate, usually "Kemithal" (sodium cyclohexenyl-allyl-thio-barbiturate), was used for induction, and analgesic drugs were given to supplement the nitrous oxide when necessary. Because previous experience with decamethonium indicated that the type of relaxation was likely to be more satisfactory for lower abdominal operations, the cases here studied were herniotomies and appendicectomies. There were 22 in all, of ages ranging from 11 to 60 (mean 33.9 years).

Relaxation.—The relaxation afforded by Ro 3-0386 was very like that produced by decamethonium. It began very shortly after the injection of the drug ; it disappeared, if anything, more abruptly than when decamethonium had been given. Some patients passed from the state requiring assistance with their breathing to apparently complete recovery in two to three minutes. Continuation of the expiratory pull of the flat muscles of the abdomen was a marked feature of this phase of the drug's action. Unlike succinylcholine, Ro 3-0386 caused no visible muscular contractions or fibrillary twitchings in man, though one or two of the early patients to whom inadequate doses were given had deviations of the eyeballs into odd positions.

Dosage.—The experimental work in the cat suggested that Ro 3-0386 was more potent than decamethonium. It soon, however, became apparent that the dose of the two drugs was about the same in man. Indeed Ro 3-0386 was slightly less potent. In adults 3–4 mg. usually depressed the respiration so much that artificial ventilation became necessary. Forceful breathing was resumed some 5–10 minutes later and more or less simultaneously the relaxation disappeared. As recommended by Paton and Zaimis (1950) for decamethonium, test doses were not used after it had become apparent that tachyphylaxis to Ro 3-0386 developed even more rapidly than with decamethonium.

Respiration.—Like decamethonium, Ro 3-0386 markedly spared the diaphragm. Intercostal paralysis developed early, and rocking-boat respiration with pronounced diaphragmatic breathing was not infrequently observed. The gasping, jerky respiration characteristic of prolonged administration of decamethonium was not observed, though, as has been noted above, this disturbance was seen in some of the dogs to which the drug was given. The absence of this complication is, however, probably due to limitation of the total dosage of the drug to an arbitrary maximum of 10 mg. and the total duration of its action to some 40 minutes. It does not imply any special merit on the part of Ro 3-0386.

Side-effects.—Ro 3-0386 had no action on the pulse rate, which remained unaltered at all rates. Usually the drug was equally without effect on the blood pressure, but in 4 of the 22 patients of this series there was some fall, the maximum decline being 25 mm. of mercury. There is little doubt that these falls in blood pressure were due to the relaxant, as they developed immediately after its administration and lasted only a few minutes. Hypotension recurred in only one of these four cases after a second dose of the drug. Neither of these features is characteristic of ganglion block, and it seems more likely that Ro 3-0386 may possess in a minority of individuals a direct depressor action comparable to that exerted by acetylcholine and many other substances with a trimethylammonium group. It is interesting that decamethonium also lowers the blood pressure occasionally in the human subject (Hunter, 1950).

Discussion

The main interest in Ro 3-0386 centres in its similarity to the relaxant decamethonium, the prototype of the depolarizing myoneural blocking agents. Thus, like decamethonium, Ro 3-0386 produces stimulation before paralysis. With both agents tetani are much better sustained than in animals paralysed to a comparable extent with *d*-tubocurarine. Both show a weak antagonism to gallamine triethiodide and are themselves rendered less toxic by the previous administration of this drug. Harmless doses of both drugs sum with one another to produce a fatal effect. The variation in species sensitivity to the drugs is somewhat similar, though the cat is more sensitive and the mouse less sensitive to Ro 3-0386 than it is to decamethonium. Both agents are successfully antagonized by compound 49-204. The action of the two drugs in the human subject is also very similar and they are approximately equipotent. The onset and disappearance of relaxation are abrupt. The same sparing of diaphragmatic respiration can be seen. There is also the same tendency to tachyphylaxis.

There is, however, something specific about the action of the two drugs. The true polymethylene compounds are antagonized to some extent by the shorter-chain polymethylene compounds, while the thiapolymethylene agents are antagonized by thiapolymethylene substances. On the other hand, hexamethonium does not antagonize the paralysis produced by Ro 3-0386, nor do Ro 3-0438 and Ro 3-0468 (the thiapentamethylene and thiahexamethylene compounds) antagonize the action of decamethonium.

Succinylcholine is the other depolarizing blocking agent in common use to-day. The pattern of its action is similar to that of decamethonium in that the onset of paralysis is abrupt and recovery rapid. It, too, sums with decamethonium and antagonizes *d*-tubocurarine and like drugs. The preliminary administration of gallamine triethiodide protects animals against dangerous doses of succinylcholine. On the other hand, this drug is

not antagonized by compound 49-204 nor yet by hexamethonium. There is, too, a very marked difference in the effective dose of succinylcholine and the other drugs. In the author's hands this agent has proved to be more than three times as toxic to mice as Ro 3-0386 and about twice as toxic as decamethonium. In cats the effective doses of decamethonium and succinylcholine are about the same. By contrast in man it takes 5–10 times the amount of succinylcholine to produce a degree of paralysis comparable to that caused by decamethonium or Ro 3-0386. It is possible that these quantitative differences are in some way related to the fact that succinylcholine depends on cholinesterase for its elimination, and it seems possible that in the human subject enough of the drug to overwhelm this enzyme must be given before paralysis can be produced. Further experiment would, however, be required to test this hypothesis.

The main point which emerges, however, from these considerations is the fact that the linking chain between the two quaternary nitrogen atoms is of importance in determining some at least of the properties of myoneural blocking agents. The intervening groups are thus more than a prop to maintain a distance of 14Å between the active trialkylammonium groups. A partial explanation of this finding may be found in the stresses in the molecule of substances of this type which result in bending of the central chain, with the result that there must be slight differences in the orientation at the muscle end-plate. In consequence only those of the same series have the required molecular configuration to displace the corresponding active myoneural blocking agents from the end-plate receptors.

The small tentative trial in the human subject of Ro 3-0386 could not provide a true indication of the place of the drug in clinical anaesthesia. The near identity of its actions with those of decamethonium, however, suggests that the two drugs might have about the same applicability, and in fact the brevity of action of Ro 3-0386 has already been put to useful purpose in electric convulsion therapy. The problem of whether Ro 3-0386 would produce cumulative effects in man like those of decamethonium has not been solved by this investigation, though rather similar effects were noted in some dogs to which the drug was given.

Summary

The drug Ro 3-0386, 5:6-dithiadecamethylene 1 : 10-bis(trimethylammonium iodide), has been shown to be a myoneural blocking agent with an action very similar to that of decamethonium, both in experimental animals and in man. It was used in 22 patients to produce relaxation for surgical operations under general anaesthesia.

The author is indebted to Roche Products Ltd. for generous supplies of this substance and for much helpful advice.

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Brit. J. Pharmacol. (1953), 8, 120.

THE EFFECT OF CORTISONE ON EXPERIMENTAL CORNEAL TUBERCULOUS LESIONS

BY

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Experimental lesions induced by the intracorneal injection of tubercle bacilli into rodents have proved useful in assessing the anti-tuberculous effect of drugs (Gardner, Rees, and Robson, 1949 ; Rees and Robson, 1950). The method seemed likely to yield information regarding the effect of cortisone in experimental tuberculosis, and the results of such an investigation are presented here.

METHODS AND MATERIAL

The intracorneal infection of the rabbit was produced by the method of Gardner *et al.* (1949). The bovine strain of *Mycobacterium tuberculosis* and the dosage employed were the same as used by these authors. The administration of cortisone was by the intravitreous route and, in the animals so treated, 0.1 ml. of a suspension containing 2.5 mg. of microcrystalline cortisone was introduced into the vitreous of the right eye twice weekly, while the same quantity of the suspending fluid only was similarly introduced into the left eye to act as a control. All rabbits were examined at weekly intervals and the extent of the lesion assessed with an arbitrary numerical scale similar to that used by Gardner *et al.* (1949).

Five rabbits were inoculated with tubercle bacilli intracorneally and divided into two groups, as follows:

Group 1.—Three rabbits—cortisone commenced on day of inoculation and discontinued on the 38th day.

Group 2.—Two rabbits—control group—untreated. This experiment was continued over a period of 56 days, and the findings are shown in Fig. 1.

A further 12 rabbits were divided into three equal groups which were inoculated with tubercle bacilli intracorneally and treated as follows:

Group 3.—Cortisone commenced on day of inoculation.

Group 4.—Cortisone commenced 14 days after inoculation.

Group 5.—Control group—untreated.

The cortisone was given as in the first experiment. Observation was maintained over a period of 42 days ; the animals were then killed and the eyes removed for histological examination. The results of this experiment are shown graphically in Fig. 2. Histological examination revealed, in both cortisone-treated and control eyes, ulcerated and caseating corneal lesions in which numerous tubercle bacilli were present. The cellular reaction in both treated and control eyes was predominantly polymorphonuclear and of similar degree in both groups.

Four more rabbits were then subjected to experimental corneal infection and treated with the antihistamine substance promethazine (phenergan). The dosage was 5 mg./kg./day given in two equal subcutaneous injections. The results of this experiment are shown in Fig. 4.

RESULTS

The administration of cortisone to the animals inoculated intracorneally with tubercle bacilli leads, as is shown in Figs. 1 and 2, to depression of the severity of the ensuing lesion. The lesions appear, as in the control animals, between the 7th–14th days, but progress much less rapidly. This effect, however, is limited to the period of cortisone administration, for after discontinuance of therapy a rapid increase in the severity of the lesion follows.

Delaying the administration of cortisone until a lesion is established at 14 days after experimental infection also produces a depression of the severity of the lesion, but to a lesser degree than that shown after immediate therapy (Fig. 2).

Illustrations of typical corneal lesions produced during these experiments are shown in Fig. 3.

The experiment with promethazine, which was investigated since, like cortisone, it has anti-allergic effects, shows quite clearly that this substance has no effect on the development of the corneal tuberculous lesion, such as was produced by cortisone (Fig. 4). It must be noted, however, that the antihistamine was given subcutaneously and that there are no data available to show whether effective concentrations of promethazine could be produced in the cornea with this method of administration, though experience with other drugs, e.g. the sulphonamides and penicillin, suggests that systemic administration of drugs is a highly effective method of reaching the cornea.

Promethazine 5 mg./kg. was injected subcutaneously into one rabbit, and three hours later the plasma and anterior chamber fluid were tested on the guinea-pig ileum for antihistamine content: both fluids contained similar small amounts of antihistamine, of the order of rather less than 1 μg./ml.

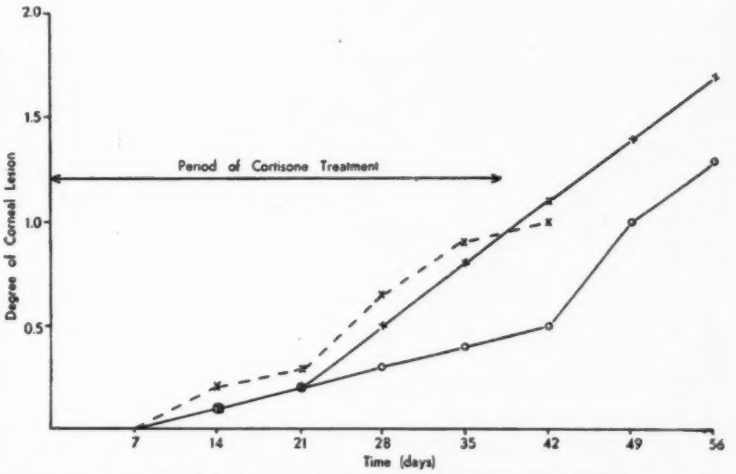


FIG. 1.—Showing the effect of cortisone treatment, started immediately after inoculation, on the progress of corneal tuberculosis in rabbits. ×——×——× Control eyes of treated animals. O——O——O Treated eyes of treated animals. ×---×---× Untreated animals. Note sudden increase in rate of development of lesions on cessation of treatment.

DISCUSSION

Cortisone affects inflammation by actions on the cellular or humoral responses to the organisms involved. There is some doubt what part is played by the inhibition of antibody (Germuth and Ottinger, 1950) or of sensitization mechanisms (Long and Miles, 1950; Woods and Wood, 1950; Long, Miles, and Perry, 1951), but the effects of cortisone on lymphoid tissue (Molomut, Spain, and Haber, 1950), granulation tissue (Ragan *et al.*, 1949), macrophage and fibroblast activity (Dougherty and Schneebeli, 1950; Spain and Molomut, 1950), and hyaluronidase action (Seifter, Baeder, and Begany, 1949; Benditt, Schiller, Wong, and Dorfman, 1950) have been clearly demonstrated. These effects are, in general, inhibitory. Lymphoid tissue is reduced in mass and appears less active histologically. The formation of granulation tissue is depressed and the macrophage and polymorph reactions are less than in control animals with either allergic or traumatic inflammations. The action of hyaluronidase on blood vessels and connective tissue is prevented. These are some of the actions of cortisone which may operate in any inflammatory situation. They may all depend, as Green (1950) has suggested, on an interference with the carbohydrate metabolism of the cells, those in a state of proliferative activity being most affected.

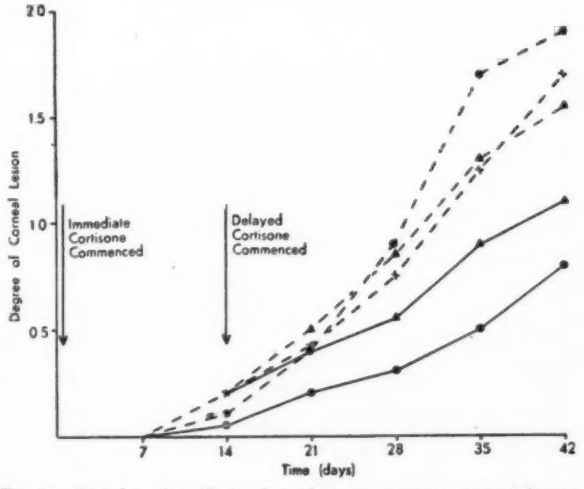
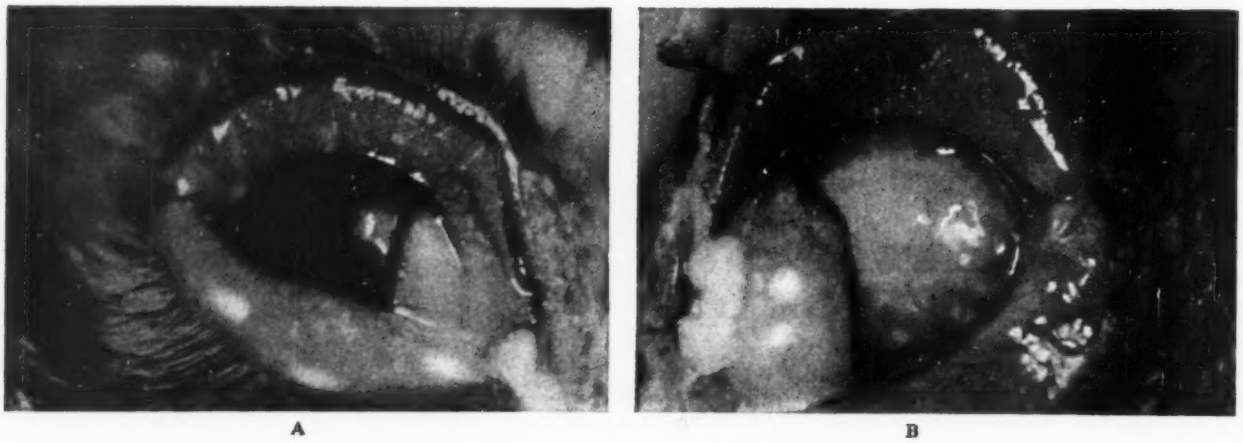


FIG. 2.—Showing the effect of cortisone treatment, started immediately after inoculation and after an interval of 14 days, on the progress of corneal tuberculosis in rabbits. ●---●---● ▲---▲---▲ Control eyes of treated animals. ●——●——● Eyes of animals treated immediately after inoculation. ▲——▲——▲ Eyes of animals in which treatment was started 14 days after inoculation. ×---×---× Untreated animals.

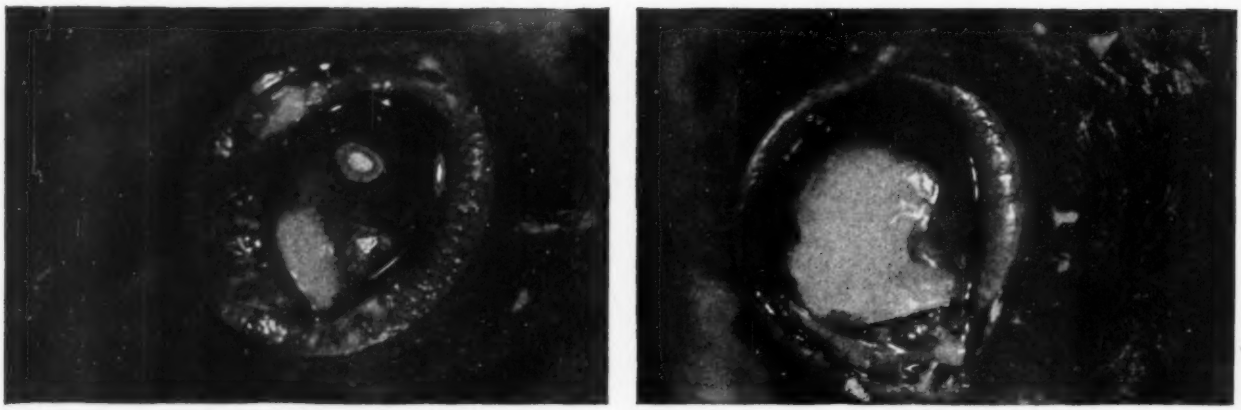
Work on experimental tuberculous infection has suggested that the sum total of the action of cortisone is usually an enhancement of the infection. Thus Hart and Rees (1950) describe an extension of previously fibrotic and static tuberculous lesions in mice following cortisone administration. Similar chronic lesions in rats were not usually fatal, but there was a considerable mortality among a cortisone-treated group (Michael, Cummings, and Bloom, 1950). Bunn and Drobeck (1952), using rabbits infected with tubercle bacilli by inoculation of organisms into the anterior chamber of the eye, found that A.C.T.H. and, to a lesser extent, cortisone, administered at, or shortly after, the time of infection, produced a rapid enhancement of the subsequent lesion.

Enhancement of experimental infections by cortisone has also been reported in poliomyelitis (Shwartzman, 1950).

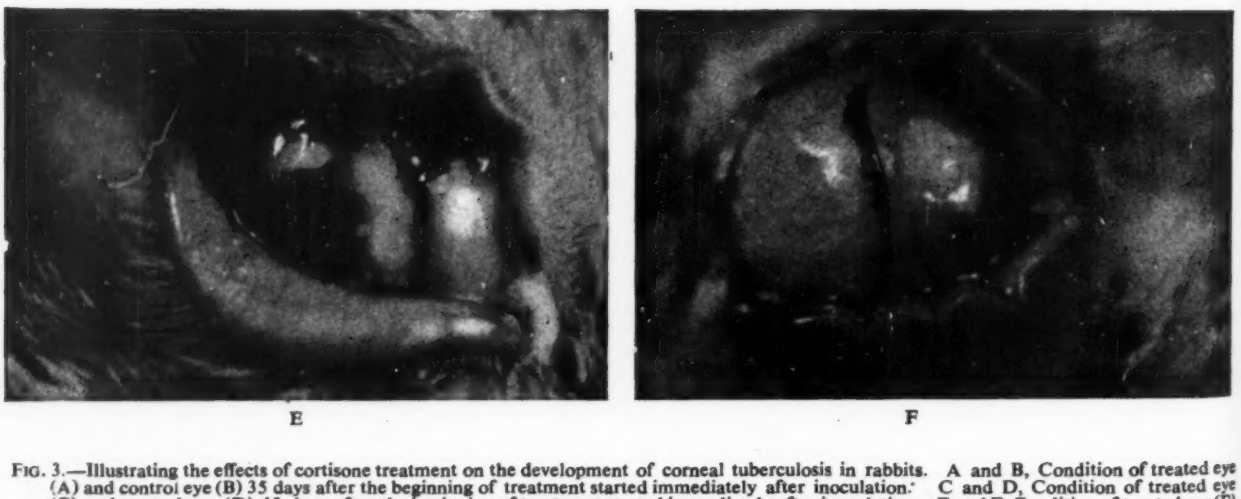
There is, however, evidence that cortisone can be of considerable value in decreasing the extent and inflammatory reaction of acute tuberculous lesions, notably in the larynx (Le Maistré and Tompsett, 1951). In man, local treatment with cortisone has produced beneficial effects in a number of inflammatory ocular conditions (Gordon and McLean, 1950; Olsen *et al.*, 1950;



A B



C D



E F

FIG. 3.—Illustrating the effects of cortisone treatment on the development of corneal tuberculosis in rabbits. A and B, Condition of treated eye (A) and control eye (B) 35 days after the beginning of treatment started immediately after inoculation. C and D, Condition of treated eye (C) and control eye (D) 45 days after the beginning of treatment started immediately after inoculation. E and F, Condition of treated eye (E) and control eye (F) 21 days after the beginning of treatment which was started 14 days after inoculation.

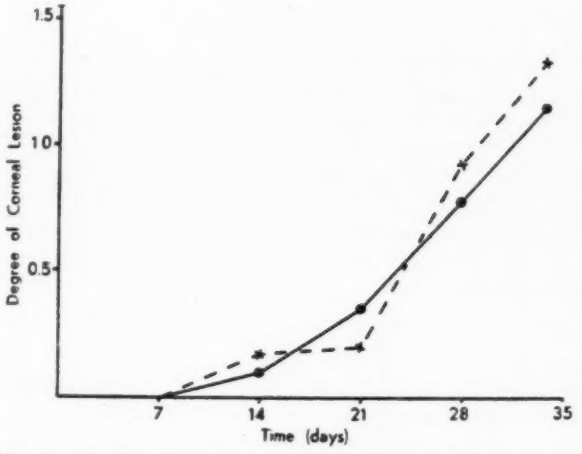


FIG 4.—Showing the absence of effect of promethazine on the development of corneal tuberculosis in rabbits. ●——●——● Eyes of animals treated with promethazine. ×---×---× Eyes of untreated animals.

Harvey, Howard, and Kattus, 1950). Here, however, there were somewhat different circumstances in that most of them were inflammatory states not associated with the presence of specific organisms in the tissues. In a few tuberculous cases there was temporary "improvement" as assessed largely by the degree of vascularity of the parts (Harvey *et al.*, 1950), but Jones and Meyer (1950) have demonstrated experimentally that cortisone will also inhibit post-traumatic vascularization of the cornea.

A great diversity in the results of the investigation on the effects of cortisone in experimental tuberculous infections is shown in the literature. Thus Spain and Molomut (1950) showed an increase in the number and extent of tuberculous lesions in guinea-pigs treated with cortisone, while Le Maistré and Tompsett (1951), also investigating experimental tuberculous lesions in these animals, found no evidence of the enhancement of such lesions by large doses of cortisone or A.C.T.H.

The evidence regarding the effect of cortisone and A.C.T.H. in experimental tuberculosis is reviewed by Dye *et al.* (1952). It is apparent that the data are rather contradictory and that no clear conclusion can be reached at this stage.

The present report of an inhibitory effect by cortisone on experimental tuberculous infection is apparently opposed to many of the results of similar work, but there are important differences in the experimental conditions. Many reports quoted deal with visceral infections often with markedly granulomatous lesions. We produced a slowly progressive local primary infection.

Histological examination of the corneae in both cortisone-treated and control animals revealed no differences in the appearances of the inflammatory reactions in these groups. Local necrosis and a moderate, predominantly polymorphonuclear, cellular response was seen in all the animals. The depression of the extension of the lesion in the cortisone-treated animals would therefore appear to result from the fact that in the cornea, a tough and avascular tissue, limitation of the extent of the infective lesion depends much more upon the plasticity of the ground substance than upon a granulomatous reaction. The spread of organisms will require increased plasticity if not liquefaction. Cortisone, by rendering the ground substance resistant to enzymatic action, may thus restrict the size of the lesion.

We believe that this is a reasonable, if at present speculative, explanation of our findings. Its consideration has at least stressed the importance of the tissues involved in deciding the effect of cortisone on experimental lesions.

SUMMARY

The effects of cortisone upon experimental infection with bovine tubercle bacilli of the rabbit's cornea has been studied, and a depressant effect upon the severity of the lesion has been observed. Such a depressant effect is not produced by the systemic administration of an antihistamine (promethazine).

The possible mechanisms of this action are discussed.

We should like to express our thanks to Dr. M. Roberts for assays of promethazine and to the Medical Research Council for a grant (to J. M. R.) which defrayed in part the expenses of this investigation. The cortisone used in this work was provided from a generous gift made jointly to the Medical Research Council and the Nuffield Foundation by Merck & Co., Inc.

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THE USE OF THE CHICK EMBRYO IN THE SEARCH FOR ANTITUBERCULOUS DRUGS

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The need, in searching for new antituberculous drugs, for a simple screening test capable of giving results more in accord with those obtained in animals than the ordinary *in vitro* test has been emphasized repeatedly (e.g., Feldman, 1946). Lee and Stavitsky (1947) described the use of the chick embryo for the purpose, using a virulent strain of the human tubercle bacillus injected intravenously, with assessment of the effect of drugs by histological examination of the liver. Intravenous injection of the embryo is technically difficult, the associated trauma causes the death of a considerable proportion of embryos, and the histological assessment is time-consuming. These factors make the method unsuitable for the examination of large numbers of compounds. It has been found (Francis, 1946 ; Francis, Peters, and Davies, 1947) that if streptococci are placed on the chorio-allantois of the chick-embryo, drugs with only "antiseptic action," such as acriflavine, have little action if injected into the yolk-sac, whilst penicillin and the sulphonamides are effective. An attempt was therefore made to apply this simplified technique in the search for antituberculous drugs. In preliminary unpublished experiments it was found that virulent mammalian tubercle bacilli produced much larger lesions than did avirulent bacilli, *Mycobacterium johnei,* or the vole acid-fast bacillus. These results would be expected, but it was surprising to find that virulent or avirulent avian bacilli produced only small lesions. The relatively low virulence of the avian strain was confirmed when it was found that after the intravenous injection of 1 mg. of moist bacilli a virulent avian strain took on the average 6.6 days to kill chick embryos and a virulent bovine strain 3.8 days. At the time, however, the lesions produced by virulent mammalian strains on the chorio-allantois were not considered sufficiently regular for routine testing. *Myco. phlei* and *Myco. butyricum* grew in the chorio-allantois but did not give satisfactory responses to known drugs. A series of tests with the avirulent human strain L.48 (National Collection of Type Cultures) showed that a suspension of 0.5 mg. of a 10- to 12-day culture on Löwenstein's medium multiplied well five days after inoculation on the chorio-allantois and a reduction of growth could be demonstrated with the standard antituberculous drugs. Later the avian strain S.H.I. was found to be more sensitive towards certain drugs in the test, and is now used in place of strain L.48.

METHODS

The following routine procedure has been adopted after various trials. Twelve-day-old chick embryos, prepared by the method of Beveridge and Burnet (1946), receive on the chorio-allantois an inoculation of 0.5 mg. (moist weight) of a strain of the avian bacillus (S.H.I.) previously grown for 12 days on Löwenstein's medium. The infecting dose of organisms is suspended by grinding in a ball mill for a short time (Martin, 1946) in 0.1 ml. of 5% broth-saline, and delivered by the McLintock automatic syringe used for tuberculin-testing cattle (McLintock, 1948). About 20 minutes later an aqueous solution or suspension of a drug is injected into the yolk-sac, with a 14-gauge needle, through a small hole drilled in the shell towards the narrow end of the egg. Ten eggs are used per group and a single dose of 18 mg. of drug per egg is given. Should this prove toxic, the dose is reduced to 9, 3, or 1 mg. in subsequent experiments. After five days' incubation the membranes from each group are harvested and ground in saline, using 0.6 ml. per membrane. The suspensions are randomized, and smears made and stained. The number of acid-fast organisms is then assessed as described below and the results obtained with the various compounds compared with the controls. A standard "active" substance is always included, usually *p*-aminosalicylic acid.

The following notation is used for the assessment of smears:

0.5 Less than five acid-fast bacilli in most fields.
1.0 10–20 acid-fast bacilli in most fields.
1.5 Few acid-fast bacilli in most fields.
2.0 Moderate number of acid-fast bacilli in all fields.
2.5 Fairly numerous acid-fast bacilli in all fields.
3.0 Numerous acid-fast bacilli in all fields.

TABLE I

ASSESSMENT BY TWO OBSERVERS OF FOUR SERIES OF SMEARS PREPARED FROM FALLING TWOFOLD DILUTIONS, IN CHORIO-ALLANTOIC SUSPENSIONS, FROM AN ORIGINAL SUSPENSION WITH AN ASSESSMENT OF 2.5

	1/1	1/2	1/4	1/8	1/16	1/32
Observer A	2·0 2·0 2·5 2·5	1·5 2·0 2·0 2·0	1·0 1·5 1·5 1·5	0·5 1·0 1·0 1·0	0·5–0 0·5 0·5 0·5	0·5–0 0·5–0 0·5–0 0·5
Average	2·25	1·88	1·38	0·88	0·44	0·31
Observer B	2·5 2·5 2·5 2·5	2·0 2·0 2·0 2·0	1·5 2·0 2·0 2·0	1·0 1·5 1·5 1·5	0·5 1·0 1·0 1·0	0·5 0·5 0·5 1·0
Average	2·5	2·0	1·88	1·38	0·88	0·63
Average of averages	2·38	1·94	1·63	1·13	0·66	0·47

It is shown below that differences of "0.5" in the assessment correspond to approximate twofold differences in the number of organisms.

Various trials were performed (with strain L.48) to test the accuracy of the method of assessment. Two observers made the same reading on 266 of 366 smears, and the readings differed by plus or minus 1.0 on only four occasions. They had, however, previously compared readings, and about the same error was made by one person on re-reading smears or when duplicate smears were made from a series of suspensions. The error was not increased if suspensions were diluted with an equal volume of saline before the smears were made. This was because a standard background was chosen for assessing the number of organisms. When, however, falling twofold dilutions of a suspension having an initial reading of 2.5 were prepared in a suspension of normal chorio-allantoic membrane, decreasing scores were obtained. All smears were assessed by two observers and the readings are shown in Table I. It will be seen from this table that one observer was reading higher than the other, but otherwise there was good agreement, and the average results show that our notations of 2.5, 2.0, etc., correspond approximately to twofold differences in the number of organisms. Smears from eggs kept in the refrigerator gave readings of about 0.5, and so a reading of 2.5 indicates that there had been a thirtyfold multiplication of organisms.

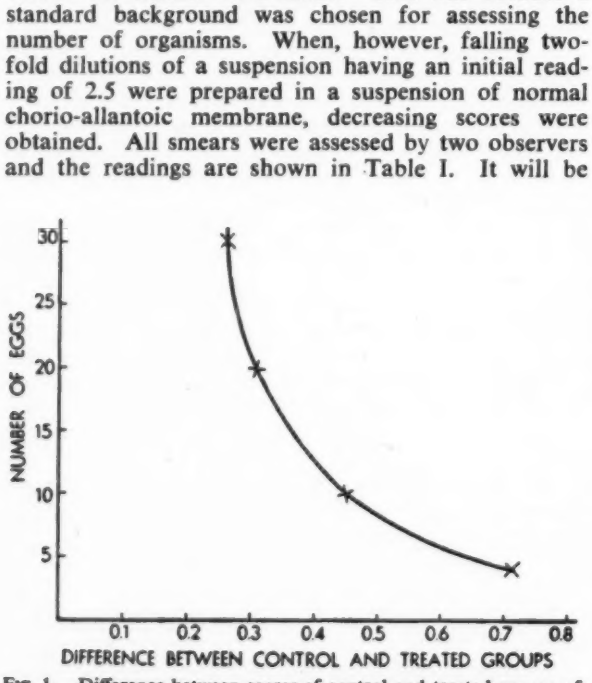


FIG. 1.—Difference between scores of control and treated groups of various sizes required for significance.

From an examination of the results of a number of tests our colleague, Dr. O. L. Davies, was able to supply the information for us to construct the curve shown in Fig. 1, and this is now used to decide whether any result is significant. It will be seen that, with a difference of 0.7 in the average readings between the control and treated groups, only five eggs are required for significance, and, with an average difference of 0.3, 21 eggs. A compound is not, however, reported as "active" unless it produces a significant effect in two, or usually three, tests.

Originally the number of organisms on the chorio-allantois of dead as well as live embryos were estimated, and as a rule the results corresponded with those in the live embryos. Presumably this is because most drugs with only an "antiseptic" action cannot diffuse out of the yolk-sac even after death of the embryo. However, a number of compounds have been observed to give spurious positive results in dead embryos only, and "effects" in dead embryos are now disregarded.

Choice of Test Organism.—Routine tests were carried out for some time using the strain L.48. However, comparative tests with a number of strains of mycobacteria showed that the virulent avian strain S.H.I. was rather more sensitive in *in vitro* tests towards compounds known to show antituberculous activity in animals (Table II). It was then shown that the S.H.I. strain was slightly but consistently more sensitive to the thiosemicarbazone No. 6057, *p*-aminosalicylic acid, and to streptomycin in the embryo, and it was decided to use this strain in preference to strain L.48. It will also be seen from Table II that the saprophytic mycobacteria were much more resistant than the tubercle bacilli to the thiosemicarbazones and *p*-aminosalicylic acid. This would explain the insusceptibility of these organisms to some of the known antituberculous drugs in the chick embryo.

RESULTS

The action of known antituberculous drugs is shown in Table III. All are active, but it is clear that the embryo test does not place these compounds in the same order as they are placed by tests in animals (cf. the weak action of streptomycin).

Up to the present, about 700 speculative compounds have been examined in the chick embryo of which some 50 have shown a signi-

TABLE II

DILUTIONS OF VARIOUS SUBSTANCES WHICH CAUSE 50% INHIBITION OF DIFFERENT STRAINS OF ACID-FAST BACTERIA ON LOWENSTEIN'S MEDIUM

(All figures × 10^{-6} except those marked by an asterisk, which are × 10^{-3})

	Human Tubercle Bacilli—Decreasing Order of Virulence from Left to Right						Virulent Bovine Tubercle Bacilli A.N.5	Avian Tubercle Bacilli			Saprophytes	
	905	Human "C"	H.37 (Blackley)	H.37 Ra	Saranac	L.48		S.H.I. (Virulent)	Sheard (Virulent)	Atten. A/30	*M. phlei*	*M. butyricum*
4:4'-Diaminodiphenyl sulphone	0·06	0·07	0·5	0·06	0·08	0·01	3·8	0·06	0·5	0·09	0·06	0·05
Streptomycin	1·2	0·7	1·0	1·7	0·58	0·15	4·7	0·1	0·2	0·6*	1·3	0·9
Thiosemicarbazone† No. 8388	3·04	2·2	4·2	1,590	4·7	0·03	8·6	0·6	0·07	4·6*	2·4*	0·25*
Thiosemicarbazone† No. 6057	476	233	824	1,590	662	0·36	426	2·9	0·73	6·0*	1·4*	0·25*
p-Aminosalicylic acid ..	181	127	662	307	209	0·02	1,590	0·01	0·36	0·49	1·0*	0·25*

The figures are the average of 3 tests. The main purpose of the test was to compare the reaction of different strains, so the partial loss of activity of streptomycin during inspissation of Lowenstein's medium was not important.

† See Table III for formulae.

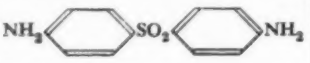
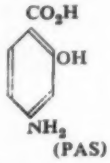
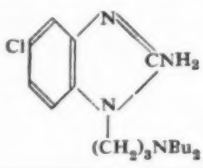
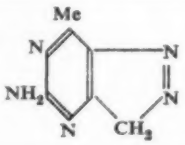
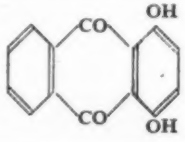
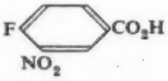
ficant positive effect. A selection of these latter compounds is given in the first part of Table III. The "active" compounds have been examined in the mouse by the method described by Martin (1946), and with the exception of No. 7438 none showed a significant degree of activity in this animal. It is evident that many more compounds are active in the embryo than in the mouse. With some drugs this may be due to rapid excretion in the mouse, or to a higher relative toxicity in this species. Nevertheless it is of interest that the last five compounds recorded in Table III, selected because of their structural relationship with *p*-aminosalicylic acid, are all inactive both in the mouse and chick embryo assays. Further, the use of the embryo test has led to the discovery of "activity" in the mouse in one novel chemical type. This relates in particular to compound No. 7438 (6-amino-4-methyl-2:3:5:7-tetra-azaindene) originally prepared in these laboratories by Dr. F. L. Rose for general investigation as an agent likely to interfere either with the synthesis or metabolism of purines, to which it is structurally related. This substance has been examined in about 100 embryos, and at a dose level of 6 mg. the mean difference of score between treated and control subjects was 0.39, which is highly significant. As a result, extensive investigations with compound No. 7438 have been carried out in several species of experimental animals, and the results will form the basis of later and more detailed communications. For the present purpose, it is sufficient to report that the mean survival time of mice infected intravenously with 0.75 mg. of *Myco. tuberculosis* (strain 905), and each given 10 mg. of the drug in the food daily, was extended by 6.3 days beyond that of a similarly infected but untreated control group. An increase in survival time of 2.8 days (at $p=0.05$) was required for significance in this particular test.

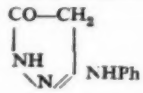
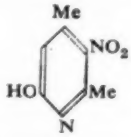
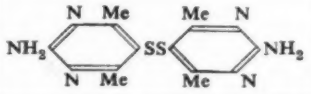
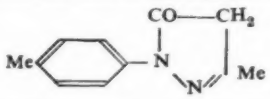
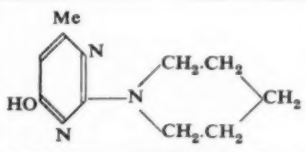
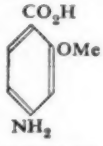
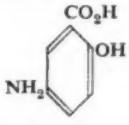
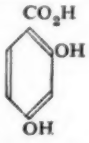
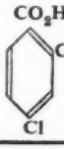
DISCUSSION

The test described in this paper detects the "activity" of the known antituberculous drugs and has led to the recognition of one new type of compound active against tuberculosis in animals. The test does not place these compounds in the same order of activity as they are placed by tests in animals. This is not surprising, since it has been shown (Hoggarth and Martin, 1950) that even in mice relative activity is frequently dependent upon the precise conditions under which the test is conducted. Generally, only one dose of compound has to be given and an answer is obtained after five days. These are important advantages over any test in animals, as the number of compounds that can be examined depends on the time, and amount of compound required, for each test. The test has the disadvantage that many compounds active in the chick embryo are inactive in the mouse, but it is still a better method of screening compounds than *in vitro* tests. This is probably because, unlike the latter, it eliminates compounds which do not pass through the living membranes without killing the embryo. The importance of giving the infective agent by one route and the drug by another is illustrated by the results of Francis (unpublished), who showed that when acriflavine, proflavine, or Nitroakridine (Hurst, Peters, and Melvin, 1950; Rasmussen and Stokes, 1951) were injected on to the chorio-allantois or even into the allantoic cavity they significantly reduced the number of pocks produced by the fowl-pox virus on the chorio-allantois, but they were quite inactive when inoculated into the yolk-sac. Lee and Stavitsky (1947) injected embryos

TABLE III

THE ACTIVITY OF VARIOUS COMPOUNDS AGAINST A TUBERCULOUS INFECTION IN THE CHICK EMBRYO

No.	Compound	Dose (mg. per Embryo)	No. of Embryos	Mean Score†
371		0·11 0·33 1·0	13 20 16	0·77* 0·8* 0·45*
8005	 (PAS)	3 9	9 14	0·72* 0·76*
—	Streptomycin	1 3 6	22 25 18	0·06 0·39* 0·63*
6057		1	281	0·76*
6082		1 3	29 29	0·31* 0·23
8388		1	4	0·7*
	Sodium tauroglycocholate	9 18	8 17	0·82* 0·65*
	Sodium deoxycholate	9 18	13 8	0·59* 1·15*
5330		1 3	13 19	0·26 0·32*
7438		6	108	0·39*
7697		9	21	0·36*
8092		3 9	5 4	0·3 1·03*

No.	Compound	Dose (mg. per Embryo)	No. of Embryos	Mean Score†
9102		18 18	28 25	0·29* 0·63*
9115		18	28	0·58*
9313		9 18	12 2	0·83* −0·6
9432		18	16	0·38*
9696		18	17	0·51*
1871		18	6	0·26
8009		18	9	−0·08
8290		9	7	−0·42
8736		18 27	3 5	0·59 0·07
8738		18	9	−0·27

† By "mean score" is meant the difference between the mean scores of treated and control groups, and an asterisk indicates that the difference is significant.

intravenously and gave drugs into the yolk-sac or on to the chorio-allantois, so their technique satisfied this criticism although it is considered to be unsuitable for the routine screening of compounds. Emmart (1946) reported experiments which purported to show the *in vivo* activity of compounds in the chick embryo. The dose of compound that killed 50% of embryos was mixed with 0.2 ml. of a suspension of tubercle bacilli, incubated overnight, and then inoculated on to the chorio-allantois. Highly significant reductions were obtained in the number of pocks which developed, but the technique does not provide a true test of activity *in vivo*.

The proportion of compounds showing a positive effect in routine testing (i.e., 50 in 700) is of the same order as that found in similar tests with other infecting organisms. Thus we have found that, with *Salm. dublin* as infecting agent in embryos, 36 out of a series of 650 compounds showed a significant positive effect, with *Corynebacterium pyogenes* 59 out of 919, and with the causal organism of fowl-pox 8 out of 800. In these tests, organisms were injected on to the chorio-allantois and drugs into the yolk-sac. With rickettsiae both drug and organism are often injected into the yolk-sac (Francis, 1952), but, as already stated, this is considered to reduce the significance of the test. It is considered that the test using tubercle bacilli has the same general significance as tests against other organisms commonly performed in the chick embryo, and constitutes a useful step in the search for antituberculous drugs.

Summary

1. A technique is described for testing the antituberculous activity of speculative compounds in which the chorio-allantoic membrane of the chick embryo is infected with an avian strain of the tubercle bacillus. A standard dose of a compound (reduced in subsequent tests, if toxic) is injected into the yolk-sac and the effect is measured by examination of smears made from the ground membrane after five days' incubation.

2. The test detects the activity of the standard antituberculous drugs, but gives positive results with more compounds than can be shown to have " activity " in animals. This is true of tests against other organisms in the chick embryo, and the test against tubercle bacilli is considered to have the same general validity as routine screening tests commonly used against other organisms in the chick embryo and to constitute a useful step in the search for antituberculous drugs. It has led to the discovery of the antituberculous activity of a new type of compound (6-amino-4-methyl-2:3:5:7-tetra-azaindene).

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EXPERIMENTAL OBSERVATIONS ON A TEST FOR SYNOVIAL PERMEABILITY

BY

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The possibility of discovering therapeutic agents which have an action on arthritis like that of cortisone naturally depends on the availability of suitable tests on laboratory animals. Methods which measure synovial permeability, which is presumably an important factor in arthritis, have been known for many years. In one of these methods (Seifter, Baeder, and Begany, 1949) the permeability of the synovial membrane under different experimental conditions was tested by injecting phenol red into the talo-crural articulation, and then measuring its output in the urine. Synovial permeability was increased by hyaluronidase ; it was decreased by cortical extracts, steroid compounds, or other compounds which possessed an action against arthritis (Bertolani, Lorenzini, and Bonati, 1951 ; Bianchi and Meli, 1952 ; Lorenzini, Bonati, and Bertolani, 1951 ; Thieblot, Simon, Laforet, and Berthelay, 1951 ; Seifter *et al.*, 1950). This test, which depends on the intra-articular injection of phenol red, does not involve synovial permeability alone. The appearance of phenol red in the urine also depends on the state of the interstitial tissue, of the capillaries, and of the kidney.

The method used by Coste, Bourel, and Delbarre (1951), which is based on the rate of liquid entering a rabbit's articulation, is independent of the action of the kidneys. However, it still involves not only the synovial membrane but also the tissues and capillaries, whereas for the clinical evaluation of anti-arthritic drugs attention should especially be centred upon their action on the synovial membrane. For this reason, several drugs possessing anti-arthritic activity have been studied by the intra-articular injection test, and by a modification of it devised to identify their exact site of action.

METHODS

Male rabbits of about 2 kg. weight were anaesthetized by intravenous injection of 40 mg./kg. of 5-5' *iso*amylethylbarbituric acid (Ethamyl Zambeletti). They were placed on their backs and a soft rubber catheter was inserted through the urethra into the bladder, which was washed with physiological saline. The catheter was retained there for the duration of the experiment. It was necessary to keep the animal completely anaesthetized and the abdominal muscles perfectly relaxed by administering ether occasionally, in order to avoid expulsion of the catheter. This did not affect kidney function or the elimination of phenol red.

In the first group of experiments 0.25 ml. of a 0.5% (w/v) solution of phenol red in physiological saline was injected into the right talo-crural articulation with an intradermal needle (No. 18). Immediately after the injection the right paw was extended and flexed five times so as to facilitate the spreading of the dye. In order to avoid any disturbances in the general circulation, the right posterior paw was fixed by a noose placed caudal to the articulation.

The bladder was emptied after 1, 2, and 3 hours, and was washed each time with about 90 ml. of physiological saline. Each sample was then made up to 100 ml. As the samples were frequently shown to contain blood, by the benzidine test, they were submitted to deproteinization before measuring their dye content. Then 0.08 ml. of a 45% (w/v) $ZnSO_4$ solution and 0.15 ml. of 1 N-NaOH were added to 10 ml. of liquid. After boiling for 3 minutes the solution was filtered through a sintered glass filter (Jena 3G4) and made up to 20 ml. A few drops of 3 N-NaOH were added and the red colour which developed was read on a Pulfrick spectrophotometer, using filter S.55. The quantity of the dye excreted was calculated from a standard curve.

In a second group of experiments 0.25 ml. of a 0.5% phenol red solution in physiological saline was injected into the periarticular connective tissue. The phenol red was then determined in the urine as previously described.

In a third group of animals the recovery of phenol red in the urine was followed after intravenous injection of 5 ml. of a 0.025% solution of the dye.

Distribution of Phenol Red between Blood and Tissue.—Finally another group of experiments was performed in order to measure the distribution of phenol red between blood and tissue fluid. The rabbits were anaesthetized by intravenous injection of

ethylurethane (1 g./kg.) and a ligature was tied round the renal pedicles in order to eliminate renal excretion. Phenol red 5 mg./kg. was then injected intravenously and the plasma concentration of the dye was measured during the next 2–3 hours. Hence the diffusion rate of phenol red from the blood to the tissues could be estimated, assuming that diffusion across the capillary membrane is a purely physical process. 3 ml. of blood was withdrawn at 5, 35, 65, 125, and 185 minutes after the injection, and was mixed immediately with 0.3 ml. of a 1% sodium oxalate solution in physiological saline. After centrifuging, the plasma was diluted tenfold with distilled water, a few drops of 3 N-NaOH were added, and the intensity of the colour developed was read as usual at 550 mμ. The solution did not need to be deproteinized, as haemoglobin was not usually present; even if it were occasionally present in small amounts, after dilution and alkalinization it does not interfere with measurements at 550 mμ.

If a substance diffuses passively from plasma to the intercellular tissue fluid through the capillary wall, considered as a uniform physical membrane, the concentration of this substance (C) should decrease in the plasma according to the following equation:

$$C = C_o \frac{V_S}{V_t + V_s} + C_o \frac{V_t}{V_t + V_s} e - kt\,(1 + V_s/V_t) \qquad (1)$$

where C_o is the concentration at zero time, V_s is the volume of plasma, V_t the volume of the tissue fluid, and K is the coefficient of diffusion of the substance through the capillary wall.

It is possible to transform this equation into a logarithmic one:

$$\ln (C - C_oA) = \ln C_oB - \frac{kt}{B} \qquad (2)$$

where $A = \frac{V_S}{V_t + V_s}$, $B = \frac{V_t}{V_t + V_s}$, and $C\text{–}C_oA$ is the difference between C at a time and C after complete equilibrium.

From each experiment plasma concentrations are obtained 5, 35, 65, 125, and 185 minutes after the injection; the course of the plasma concentration curve obtained is reproduced in Fig. 1. If the logarithms of the difference between the concentrations after 3, 35, and 65 minutes and that at 125 minutes are plotted on the ordinate and the corresponding times on the abscissa, theoretically a straight line should be obtained (Fig. 2). As shown in Fig. 1, the plasma concentration of dye at 125 minutes after injection represents nearly an equilibrium value.

If B is constant, the slope of this line, represented by K/B, is related to the diffusion coefficient of phenol red through the capillaries, and, if K is constant, the slope of this line is related to the ratio between tissue fluid volume and plasma-plus-tissue-fluid volume. K/B was calculated statistically for each experiment and for each group of animals.

The distribution of phenol red between blood and tissue fluid is presumably more complex than we have assumed; nevertheless this simple procedure does permit us to estimate differences in the distribution rate between treated and control animals.

All the animals except the controls were previously treated for four days with the drugs under experiment

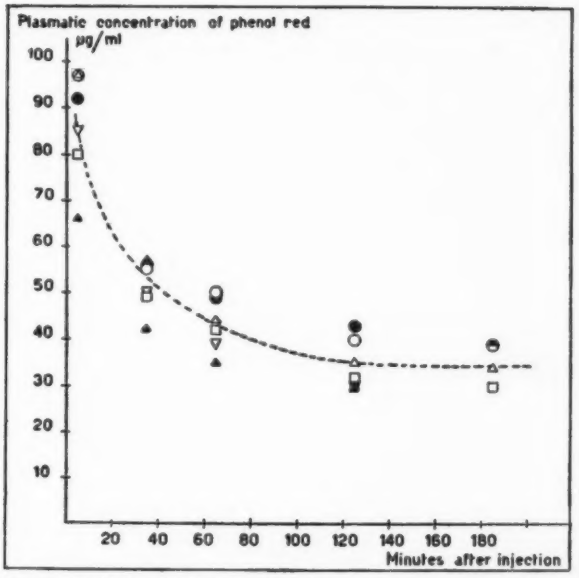

FIG. 1.—Rabbits with tied renal pedicles. Plasma concentration of phenol red plotted against time in minutes after intravenous injection of 5 mg./kg. of the dye. The different types of symbols designate plasma concentration of dye in different animals.

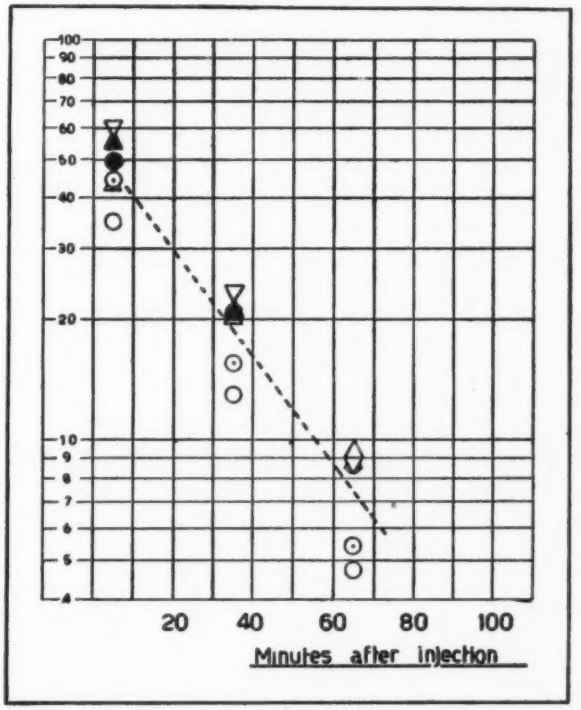

FIG. 2.—Rabbits with tied renal pedicles. Differences between plasma concentration of phenol red at 5, 35, 65 minutes and concentration at 125 minutes after intravenous injection of 5 mg./kg. of the dye plotted against time in minutes. The different types of symbols designate results in different animals.

cortisone acetate, pregnenolone, 21-acetoxypregnenolone, desoxycorticosterone acetate (DOCA), benzoylcarbinol acetate (Logemann and Giraldi, 1951 ; structural formula: C_6H_5-CO-CH_2-O-$COCH_3$), and sodium salicylate. These products were injected intraperitoneally, 300 mg./kg. in all (75 mg./kg. per day). DOCA was injected intraperitoneally, 20 mg./kg. in all (5 mg./kg. per day). A commercially available suspension of cortisone (Cortone Merck) was used. All the other products were suspended at a 2% (w/v) concentration in a 10% (w/v) gum acacia solution.

The Intra-articular Injection of Phenol Red.—Some observations must be made about the technique described by Seifter *et al.* (1949) for intra-articular injection of phenol red. The noose fixing the paw must always be put caudal to the articulation when the injection is made, because a slight pressure on the upper part retards absorption. When a pressure of 50 cm. H_2O was exerted by a sphygmomanometer cuff placed cranial to the articulation. the phenol red absorbed was 37% less in one hour and 22% less in three hours than what it was with the articulation not constricted (mean values of four experiments).

The temperature must be maintained constant throughout experiments, as the circulation also varies with the external temperature ; the phenol red absorbed by the articulation of animals maintained at a constant temperature of 17° C. throughout an experiment was 39% less in one hour and 22% less in three hours than what it was in animals maintained at a temperature of 21° C. (mean values of four experiments).

Animals cannot be used more than once. When the same animal was used ten days after a first experiment, the absorption of phenol red was found to be 22% less in one hour and 28% less in three hours (mean values of four experiments) than it had been in the first experiment. In the second test the injection was made into the articulation not previously used. These precautions were always observed in all tests.

Seifter, Baeder, and Blumenthal (1952) have also described the factors influencing synovial membrane permeability.

Results

The compounds studied have been divided into three groups, according to their type of action (Table I).

Group 1 (Cortisone, Na salicylate, benzoylcarbinolacetate)

These three compounds act on the synovial membrane only. Whereas absorption of phenol red from the articular cavity was partially inhibited (this result is statistically uncertain with cortisone) absorption after subcutaneous injection was not altered, nor was passage of phenol red through the capillaries or kidney altered.

Group 2 (Desoxycorticosterone-acetate (DOCA))

This compound acts on subcutaneous tissue, but not on the kidney, capillaries, or synovial membrane. The amount of phenol red eliminated after intra-articular injection of dye was nearly equal to that obtained after injection into the subcutaneous tissue near to the articulation.

Group 3 (Pregnenolone and 21-acetoxypregnenolone)

The strong inhibitory action of these products on the elimination of phenol red was due wholly or partially to a mechanism different from the previous one. When phenol red was injected into the blood stream less was eliminated than in the control observation. This could be explained by the inhibition of phenol red elimination through the kidneys.

For pregnenolone there is also an increase of the value K/B for equation (2). This could be explained by an increase in capillary permeability or bv a decrease in absorption capacity of tissues for phenol red (V_t).

TABLE I

RENAL EXCRETION OF PHENOL RED 1 HOUR AND 3 HOURS AFTER INTRA-ARTICULAR, PERIARTICULAR, OR INTRAVENOUS INJECTION (PERCENTAGE OF INJECTED AMOUNT)

For K/B see text. Probable deviations are quoted after each figure*

i.p. Injection of †	Intra-articular		Periarticular		Intravenous		Capillary Permeabi ity K/B 2·3
	1 hr.	3 hr.	1 hr.	3 hr.	1 hr.	3 hr.	
O (controls)	54·2± 4·4	76·8± 5·0	55·4±10·4	88·0±10·5	72·5± 7·9	86·8± 7·3	−0·0134±0·0009
Cortisone acetate ..	48·0±18·0	72·5±14·8	52·2±10·3	79·8± 6·1	—	—	—
Na salicylate	39·1± 9·5	62·7± 6·2	58·4± 6·9	85·2± 2·8	—	—	−0·0149±0·0029
Benzoylcarbinolacetate ..	38·2± 6·4	62·2± 3·9	58·1± 6·3	84·7± 4·9	—	—	−0·0154±0·0033
Desoxycorticosterone acetate	46·0± 6·4	69·4± 7·0	46·9± 5·7	82·4± 2·0	74·9± 2·9	88·8± 2·8	−0·0157±0·0019
21-Acetoxypregneno'one	20·4± 7·1	46·9± 4·2	19·1±11·7	45·0±13·6	58·4± 8·6	77·6± 7·4	−0·0139±0·0026
Pregneno'one	28·1± 7·5	60·6± 6·2	29·7± 8·1	64·8±12·5	33·1±14·5	52·9±13·3	−0·0208±0·004

* These figures are based on from 4–6 experiments for each compound, and from 6–16 experiments on the control observations.
† For amount injected, see text.

Discussion

From our experiments it appears that the decreased urinary elimination of phenol red after intra-articular injection into rabbits cannot always be explained in the same way; the experimental results may have an articular explanation or an extra-articular one (subcutaneous, renal, or circulatory).

Sodium salicylate, which has a certain anti-arthritic action, shows also a clear action on synovial permeability; on the contrary, cortisone, which is notoriously an anti-arthritic compound, shows a less evident effect on the synovial membrane. Cortisone also failed to cause any significant alteration of synovial permeability, according to Paul, Hodges, Knouse, and Wright (1952) and Hidalgo, McClure, Henderson, Whitehead, and Smyth (1952). Benzoylcarbinolacetate also had a clear action on the synovial membrane, but its therapeutic effects are uncertain. Another compound, cinchophen, used successfully in therapy, had a strong inhibitory action on renal elimination of the dye and cannot therefore be used for this test (Bianchi and Bernini, 1952). Of the other compounds investigated, none had a specific action only on the synovial membrane; this suggests that a different mechanism of action exists. The greater activity of 21-acetoxypregnenolone compared to cortisone, which was described by Seifter *et al.* (1950) and confirmed by others (Bianchi and Meli, 1952), had not the value attributed to it, as the action of the two compounds was different. In opposition to other opinions DOCA had no specific action on the permeability of the synovial membrane.

It is difficult to compare our results with those obtained with other tests based upon diffusion phenomena, such as those involving the Indian-ink intradermal diffusion or the passage of water through connective tissue membranes (Bianchi and Meli, 1952; Capraro and Meli, 1951). These tests are based on the inhibitory action of compounds on diffusion increased by hyaluronidase. The elimination of phenol red after injection into the articular cavity, as well as the antihyaluronidase activity, is influenced by other properties of compounds which modify the permeation phenomenon as shown for pregnenolone and 21-acetoxypregnenolone. For this reason, according to our opinion, a screening test for synovial permeability must not be limited to the single measurement of the excretion of phenol red injected into the articular cavity. Obviously, if the elimination of phenol red were not modified, it would be unnecessary to look further. But if elimination is delayed, then it is necessary to examine the various possible mechanisms.

Summary

1. The renal excretion of phenol red after intra-articular injection in the rabbit treated with different compounds does not depend only upon the permeability of the synovial membrane. The permeability of the connective periarticular tissue, of the capillaries, and of the renal tubules may also modify the renal excretion.

2. By using various techniques (intra-articular injection of phenol red; injection of phenol red into the periarticular connective tissue; intravenous injection of phenol red in normal rabbits; intravenous injection of phenol red in rabbits with a ligature tied round the renal pedicles) it is possible to identify the site of action of the compounds under investigation.

3. Sodium salicylate and benzoylcarbinolacetate diminished the permeability of the synovial membrane. Cortisone had an uncertain action.

4. Desoxycorticosterone acetate diminished the permeability of subcutaneous periarticular tissues.

5. Pregnenolone and 21-acetoxypregnenolone mainly decreased the renal excretion of phenol red.

I am indebted to Mr. Gianfranco Biganzoli for his technical assistance.

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Brit. J. Pharmacol. (1953), **8**, 134.

A CONTRIBUTION TO THE PHARMACOLOGY OF MOVEMENT IN THE LIVER FLUKE

BY

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Baldwin and Moyle (1949) found that nerve-muscle preparations from ascaria responded in a different manner from those of annelids. The present study has been undertaken to extend this type of comparison to include a representative of the platyhelminthes (trematoda), *Fasciola hepatica*, the liver fluke.

METHODS

The method of collecting and keeping liver flukes for the study of their movement has been described in our previous paper (Chance and Mansour, 1949).

An attempt was first made to isolate a neuromuscular preparation from the parasite consisting of one layer of muscle, thus exposing a maximum amount of tissue to the effects of the different drugs. It was found that preparations made from only one side of the muscular layer did not show any rhythmical movements on the kymograph.

An alternative preparation which was made by cutting off the oral end provided a means of eliminating the effects of the circum-oesophageal ganglia. This preparation was attached to the kymograph lever in the same way as the whole preparation. When fully relaxed, the muscle between the two attachments was stretched to about 2½ cm., and contracted to a minimum length of about 1 cm. The magnification of the lever was 1½. Fresh, active flukes of a moderate size were chosen. The preparations were completed as quickly as possible in a glass vessel containing medium at 37° C. They were then mounted in a 50-ml. organ-bath. For the composition of the medium see Chance and Mansour (1949). Drugs were added to the organ-bath, and each observation was repeated four times. Initially, movements were rhythmical, very weak, and slow, but they were soon stimulated by the weight of the lever. Addition of weights, or moving the lever up and down to induce tension stimulation, was a good method of initiating rhythmical activity. After this treatment, the majority of the preparations began to show movement of fairly constant frequency and tone for at least two hours.

For the chemical studies the parasites were dissected from the bile ducts of infested cattle, transferred to Locke's solution (maintained at a temperature of 37° C.), and quickly taken to the laboratory. There the flukes were washed in warm Locke's solution, dried between filter papers, and weighed. They remain fully active under these conditions (Chance and Mansour, 1949).

Enzyme extracts were prepared by grinding the whole parasite to a fine suspension in Krebs' Ringer. Warburg's manometric technique was employed to determine the carbon dioxide evolved on incubation with two different substrates; 0.18 M acetyl-β-methylcholine chloride and 0.36 M benzoylcholine chloride.

RESULTS

The undulating movement of the posterior part of the body is one of the characteristic movements of the normal parasite. This movement results in shortening of the body, but it is soon followed by extension. It was found that the undulatory movements of preparations from the degangliated parasites were similar to those of the whole parasite.

Acetylcholine chloride was tested on both preparations at concentrations varying from 10^{-4}–10^{-3}. The whole preparation was unaffected. Only at a concentration of 10^{-3} was a response obtained from the degangliated preparations (Fig. 1). This was a contraction, followed by cessation of normal rhythmical movement in a relaxed position, for 15 minutes, and then followed by gradual recovery. The period of depression varied from one preparation to another in intensity and duration, but was not less than 12 minutes.

Carbaminoylcholine chloride. — Carbachol, which is not readily destroyed by choline esterase, produced complete paralysis in both preparations in concentrations varying from 10^{-4} to 2×10^{-4}; the stronger solution was more dependable in causing paralysis. The effect produced a marked contraction, followed by paralysis in the relaxed position.

Eserine.—The whole and the degangliated preparations were very sensitive to the action of eserine. The response was characterized by either

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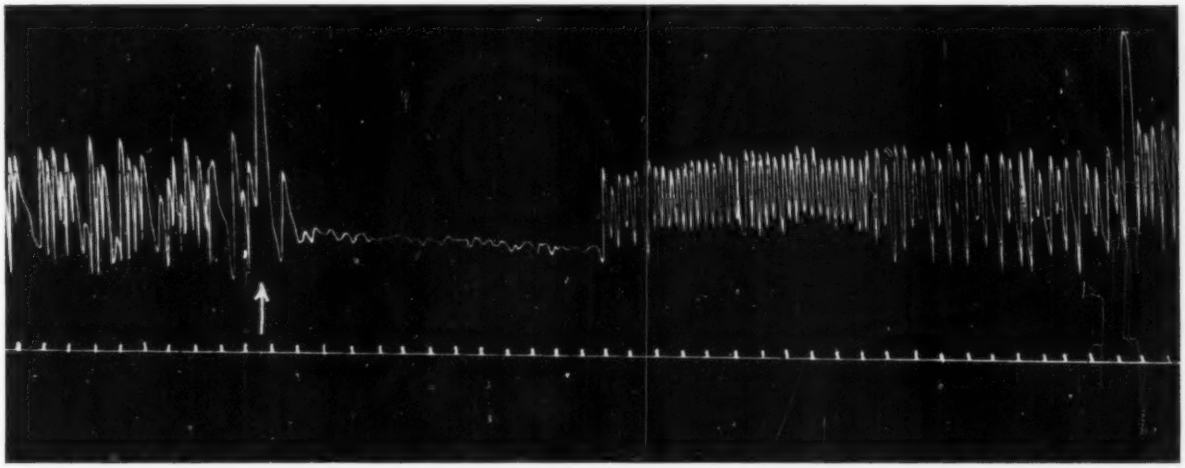

FIG. 1.—Degangliated liver fluke preparation. At the arrow acetylcholine was added, to give a concentration of 10^{-3}. Time marks minutes.

complete cessation of movement or by marked depression of amplitude and rate of rhythmical movement whilst the preparation was relaxed Preparations from the whole or degangliated parasite showed these responses at concentrations above 10^{-5}. The preparations which were not immediately paralysed by eserine were greatly affected, sometimes to the point of paralysis, if left in contact with the drug for about 90 minutes. This shows that the action of eserine is not reversible like that of acetylcholine. It also shows that eserine, like carbachol, lowers the tone and obliterates the rhythmical movement, but it does so more slowly. The two actions differ only in the rate of induction.

Eserinization at concentrations which did not paralyse the preparation was continued for 30–40 minutes, and after this time the acetylcholine was added in appropriate amounts.

The results with the degangliated preparations were as follows: In the degangliated preparation acetylcholine had no effect in the absence of eserine, even in a concentration of 500 μg./ml. (4 exps.). High concentrations of eserine (20 μg./ml. and over) had a direct depressant effect. A concentration of 10 μg./ml. had no effect by itself (13 exps.), but sensitized the preparation to acetylcholine so that a concentration of 100 μg./ml. paralysed one preparation out of 2 and 200 μg./ml. paralysed 4 preparations and depressed 1, and a concentration of 500 μg./ml. paralysed all of 5 preparations. In the presence of eserine (5 μg./ml.) one preparation was depressed and another paralysed by acetylcholine (100 μg./ml.).

In the whole preparation 1,000 μg./ml. of acetylcholine had no effect in the absence of eserine, but 200 μg./ml. depressed preparations sensitized by eserine (2–5 μg./ml.).

Higher concentrations of eserine (10–100 μg./ml.) had some depressant effect and also sensitized the muscle to acetylcholine (100–200 μg./ml.).

Cholinesterase and an Acetylcholine-like Substance.—The results of the estimations of cholinesterase activity as recorded by the evolution of carbon dioxide from the ground material of the liver fluke in the presence of different substrates is seen in Table I. Koelle and Gilman (1949) classify this enzyme in two parts: "true cholinesterase," which will hydrolyse acetyl-β-methylcholine chloride but not benzoylcholine chloride, and "pseudo-cholinesterase," which will hydrolyse benzoylcholine chloride but not acetyl-β-methylcholine chloride. The results have shown that the material from whole parasites could hydrolyse

TABLE I

HYDROLYSING CAPACITY OF THE WHOLE PARASITE TISSUE TO DIFFERENT CHOLINE ESTERS AND ITS ABILITY TO ABSORB CO_2

Substrate	Concentration in 0·5 ml.	No. of Manometers	μl. CO_2 Evolved or Absorbed/g. (Wet Weight)/hr.	Mean
Acetyl-β-methylcholine chloride	0·18 M	3	752	1,008
		3	1,024	
		5	1,256	
		5	1,000	
Benzoylcholine chloride	0·36 M	2	−80	−118
		2	−160	
		2	−64	
		3	−168	
Krebs' Ringer solution		4	−64	−136
		6	−248	
		6	−104	
		3	−96	
		2	−168	

acetyl-β-methylcholine chloride but not benzoylcholine. This fact is evidence for the presence of true cholinesterase in the liver fluke.

The presence of an acetylcholine-like substance and the synthesis of it in the material of the liver fluke was demonstrated with eserinized material from four different samples of mixed and ground fresh liver flukes. It was assayed on the guinea-pig ileum against acetylcholine as a standard. The values for the separate samples were 0.33, 0.2, 0.19, and 1.7 μg./g. wet weight. The remainder of the material was then incubated for one hour and reassayed. The following values were then obtained: 10.8, 4.0, 4.3, and 12.7 μg./g. wet weight. The activity both before and after incubation was blocked by atropine, and a sample of the material was in each instance inactivated after boiling with N/3 NaOH. It may be concluded that an acetylcholine-like substance is present and that it is synthesized by the tissue.

Nicotine.—The responses in whole and in degangliated preparations were characterized by a rapid contraction, which very gradually relaxed to half tone, and continued for a considerable time at this level. A point of interest should be noted here. Nicotine did not reproduce the action of acetylcholine because acetylcholine produced the same strong contraction which was soon followed by a marked relaxation.

Curare and Atropine.—*d*-Tubocurarine chloride and atropine sulphate were found to be without effect on the whole and on the degangliated preparations up to a concentration of 10^{-3} on the whole preparation, and five times this concentration on the degangliated parasite. The above substances were then tested for their antagonism to acetylcholine and carbaminoylcholine chloride.

Atropine sulphate or *d*-tubocurarine chloride at concentrations of 5×10^{-2} were left in contact with the preparations for 1 hour, and the drug to be tested was then added to the organ bath at a high concentration. On the whole and degangliated preparations, atropine and curare were found to cause at least partial block to the effect of acetylcholine and carbachol. The marked contraction which immediately follows the addition of acetylcholine was also abolished.

Eserine, after the usual atropinization or curarization, was tested at concentrations of 10^{-5} to 5×10^{-4}, but at all concentrations its usual effect was undiminished.

Responses to Sympathomimetic and Other Neuromuscular Drugs.—In our previous investigation amphetamine, phenylethylamine, ephedrine, and tyramine were all found to have a stimulant effect on whole parasite preparations (Chance and Mansour, 1949). The present study revealed that phenylalkylamines possess this stimulant effect, and the minimum effective concentration for each amine is enumerated below.

Stimulants: *d*-Amphetamine SO_4, 1 in 80,000; *r*-amphetamine SO_4, 1 in 20,000; *l*-amphetamine SO_4, 1 in 10,000; β-phenylethylamine HCl, 1 in 5,000; propadrine HCl, 1 in 2,000; ephedrine HCl, 1 in 2,000; tyramine HCl, 1 in 1,000; kephrine HCl, 1 in 5,000, and neosynephrine HCl, 1 in 1,000, depressed movement, and adrenaline HCl, 1 in 2,000, had no effect. All these substances constitute Group A referred to below.

Nikethamide, 1 in 1,000, and metrazol, 1 in 1,000, were without effect, but the following substances were all stimulants: Strychnine HCl, cocaine, procaine HCl, amethocaine HCl, quinidine SO_4—referred to as Group B.

Group A has been reported as resistant to the oxidative action of amine oxidase, but able to form stable complexes with this enzyme. On the other hand, catechol compounds, adrenaline, kephrine, and those with a similar pharmacological action (e.g. neosynephrine) did not show any stimulant effect. Adrenaline hydrochloride at concentrations below 2×10^{-3} did not interfere with rhythmical movement, while kephrine and neosynephrine had a depressant action.

Adrenaline is highly sensitive to destruction by amine oxidase, while ephedrine blocks its effect; it was therefore thought worth while to test the effect of adrenaline on preparations treated with ephedrine 10^{-4} for different lengths of time; it was found that, even in the presence of ephedrine, adrenaline hydrochloride had no effect.

In Group B, three local anaesthetics showed a stimulant action; cocaine was the most potent of this group. Strychnine, a convulsant to vertebrates, was a strong stimulant to the degangliated and whole parasite preparations. The type of stimulation due to strychnine was irregular.

Discussion

The rhythmical movement of the degangliated preparation was the same as that of the whole preparation, and the behaviour of the degangliated preparation in response to the drugs which have been tested was (except acetylcholine) also the same. We may conclude, therefore, that the peripheral neuromuscular mechanism possesses its own intrinsic rhythm and that the action of these drugs was on this mechanism, and was not mediated by any action on the anterior ganglia. The responses evoked by different types of

neuromuscular drugs seem to differ strikingly from those obtained with other types of helminths. It is already known that acetylcholine produces a sudden contraction, which is continuous for as long as it is in contact with annelid preparations (Fühner, 1918). On exposed ascaris muscle preparations acetylcholine produces a stimulant effect marked by a sharp rise in tone and corresponding fall of amplitude (Baldwin and Moyle, 1949). In contrast, acetylcholine relaxes the fluke and depresses its rhythmical movement after a brief contraction, but recovery is spontaneous in the presence of the drug. Moreover, eserine produces a similar effect and intensifies the action of acetylcholine on the fluke. Baldwin and Moyle (1949) were unable to demonstrate a potentiating action of eserine on the effect of acetylcholine on ascaris. The liver fluke synthesizes an acetylcholine-like substance, and is able to destroy it through the action of true cholinesterase. It seems likely, therefore, that the action of eserine is due to the presence of a cholinergic mechanism, similar in some respects to that found in mammals, which plays a part in the mechanism controlling movement.

The responses of the preparation to the different sympathomimetic amines are surprisingly in line with their stimulant action on the mammalian central nervous system, as found by Schulte *et al.* (1941), with mice in "jiggle" cages. These authors found *d*-amphetamine the most potent amine. In their tables, the substances are arranged in descending order of potency, assessed by the dose required to produce maximum activity at the peak period. The order was *d*-amphetamine, *l*-amphetamine, propadrine, phenylethylamine, ephedrine, and adrenaline. Tyramine and neosynephrine did not show any stimulant effect in any dose tolerated by mice. The order here is not very different from the action on flukes. The three amphetamine derivatives had the same order, while phenylethylamine was superior to propadrine. Tyramine showed a definite stimulant effect on the liver fluke, but it did not on the activity of mice, while neosynephrine and kephrine depressed the rhythmical movement.

Morton and Tainter (1940) and Lawrence, Morton, and Tainter (1942) made use of the hypothesis put forward by Gaddum and Kwiatkowski (1938), on the mode of action of ephedrine, to explain the mechanism of other amine oxidase inhibitors. This enzyme is responsible for the deamination of some of the sympathomimetic amines, including adrenaline. Tainter and his colleagues put tyramine, propadrine, phenylethylamine, and cocaine among the ephedrine group; while neosynephrine was put in the adrenaline group, which owe their effect to a direct action on the effector cells. From the results reported here, it can be seen that all the amines of the ephedrine group, and also cocaine, possessed a stimulant action. Yet amines of the adrenaline group were either ineffective or had a depressant action.

Amphetamine, as a representative of these sympathomimetic amines, produced its stimulant effect in the presence of nicotine and carbachol. Conversely, however, when the fluke was under the influence of any stimulant drug, the preparation was insensitive to the inhibiting action of carbachol.

Degangliated and whole preparations were highly sensitive to the stimulant action of strychnine. Nachmansohn (1941) demonstrated *in vitro* that strychnine inhibits the hydrolysis of acetylcholine by cholinesterase. He then attributed the effect of strychnine on the central nervous system to the fact that this stimulant causes an increase in the acetylcholine, and this in turn causes a marked condition of excitement. This cannot be so in the case of the fluke, since an increase of acetylcholine intrinsically produced by eserine, or extrinsically by the addition of acetylcholine itself, causes depression to the rhythmical movement, if not complete relaxation.

Most of these stimulant compounds were tried by Baldwin and Moyle (1949) on nerve-muscle preparations from ascaris. They found that cocaine possesses a paralysing action, while strychnine, ephedrine, and tyramine are innocuous to rhythmical movement of a preparation. This difference provides further evidence of the existence of important physiological and pharmacological differences between the liver fluke and ascaris.

Acetylcholine is well known to be closely connected with the motor activity of invertebrates and vertebrates (Bacq, 1949). A recent and convincing contribution in this field of study has been the work by Bülbring, Lourie, and Pardoe (1949). They demonstrated the manner in which *Trypanosoma rhodesiense* (motile protozoa) contains and synthesizes acetylcholine. It was also proved that the non-motile protozoa *Plasmodium gallinaceum* neither contained nor synthesized acetylcholine. Bülbring and Burn (1949) provided evidence to suggest that even in mammalian heart muscle, where acetylcholine is supposed to be inhibitory, the activity of atrial muscle is closely linked with the synthesis of acetylcholine.

Fasciola hepatica is a parasite well equipped for a high degree of motility. Motor activity of neuromuscular preparations has been recorded on the kymograph, even in the absence of the central ganglia in the proboscis of the parasite (Chance and Mansour, 1949). Acetylcholine and its allied compounds were found to be inhibitory to the muscular activity recorded from these preparations. In important respects, therefore, the musculature of the liver fluke resembles the heart muscle of mammals, not only in the rhythmical nature of the movement, but also in the possession of cholinergic components and in its response to acetylcholine.

The presence of cholinesterase in the fluke provides an explanation of the effects of the cholinergic drugs, the action of which was the same on the whole and degangliated preparations, and makes it likely that the absence of the effect of acetylcholine at 10^{-3} was due to slow penetration resulting in the destruction of the substance before it could reach the receptors, rather than that it has an opposing action on the ganglia.

Summary

1. A technique for the removal of the central ganglia in the liver fluke has been described and the action of drugs on this preparation has been compared with the whole animal.

2. These preparations showed rhythmical movement when suspended under tension in Ringer solution. Carbaminoylcholine chloride, acetylcholine, and eserine affected the degangliated preparation in a similar manner to the normal, either producing paralysis of movement or reducing the amplitude of the contractions.

The relaxing action of acetylcholine was temporary, but that of carbaminoylcholine chloride was long-lasting.

Eserine sensitized the preparation to the effect of acetylcholine.

3. The effect of the two choline esters was blocked by atropine or curare.

4. Rhythmical activity of the preparations of the liver fluke was stimulated by some sympathomimetic amines. There is a close similarity between this action and the action of these substances as central nervous system stimulants in mammals.

5. These results, when compared with published work on ascaria, show that these two parasites differ markedly in their reaction to the same drugs.

6. Liver flukes have been found to contain an acetylcholine-like substance in amounts equivalent to 0.19–1.7 μg./g. (wet weight) of acetylcholine.

The formation of an acetylcholine-like substance has been demonstrated *in vitro* in amounts equivalent to 4.0–12.7 μg./g. (wet weight) of acetylcholine in 1 hr. at 37° C.

The presence of true cholinesterase and the absence of pseudo-cholinesterase in the liver fluke has also been demonstrated.

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THE ANTIVIRAL ACTION OF PHENANTHRIDINIUM COMPOUNDS

BY

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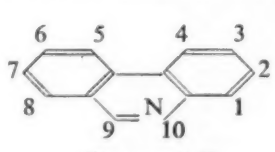
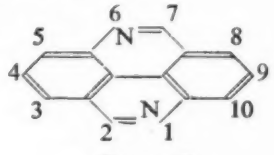
Dickinson and Codd (1952) reported that a phenanthridinium compound, 2:7-bis-(2-dihydroglyoxalinyl)-9-phenylphenanthridine hydrochloride dihydrate, inhibited the development of a bacteriophage of *Pseudomonas pyocyanea* at a much lower concentration than that having any apparent effect on the growth curve of the host. The activity was not due to the direct inactivation of free phage, and the phenomenon seemed of sufficient interest to warrant further investigations of the phenanthridines. About a hundred phenanthridines and related compounds had been prepared by our colleagues in the chemistry division of the Research Department, and these compounds were investigated for their activity against (1) Pb bacteriophage of *Ps. pyocyanea*; (2) influenza A virus in eggs (two compounds in mice); (3) Gram-positive and Gram-negative bacteria; (4) Rous sarcoma virus I in chicks (selected compounds only). The compounds were classified into eight groups, and results, summarizing those for all the tests, are given for the following 23 compounds:



Groups 1–7



Group 8

Group 1

1095.—9-Anilinophenanthridine.

1279. — 9-Anilino-10-methylphenanthridinium chloride hydrochloride.

1280. — 9-(N-Carbethoxyanilino)-10-methylphenanthridium methanesulphonate.

Group 2

Phenidium.—7 - Amino - 9 - *p* - aminophenyl-10-methylphenanthridinium chloride (Morgan and Walls, 1938).

Group 3

481. — 2:7-Diamino-10-methyl-9-phenylphenanthridinium bromide (Walls, 1945).

1602. — 2:7-Diamino-10-*n*-amyl-9-phenylphenanthridinium chloride (Watkins, 1952).

1470. — 2:7-Diamino-10-*n*-hexyl-9-phenylphenanthridinium chloride (Watkins, 1952).

1446. — 10-allyl-2:7-diamino-9-phenylphenanthridinium bromide (Watkins, 1952).

Group 4

1390.—2:7 - Diamino - 9 - *p* - aminophenyl - 10-methylphenanthridinium bromide (Walls and Whittaker, 1950).

1343. — 2:7-Bisglucosylamino-9-*p*-aminophenylphenanthridine (Walls and Whittaker, 1950).

1662. — 2:7-Diamino-10-ethyl-9-*p*-nitrophenylphenanthridinium chloride (B. Application No. 5926/52).

1601. — 2:7 - Diamino - 9 - *p* - nitrophenyl - 10-propylphenanthridinium chloride (B. Application No. 5926/52).

Group 5

2202. — 2:7-Diamino-9:10-dimethylphenanthridinium bromide (Walls, 1947).

1098.—2:7-Diamino-9-butylphenanthridine.

1120. — 2:7-Diamino-9-butyl-10-methylphenanthridine methobromide.

Group 6

1099. — 2:7-Dicarbethoxyamino-9:10-dimethylphenanthridinium methosulphate (Walls, 1947).

1097. — 9-Butyl-2:7-biscarbethoxyamino-10-methylphenanthridinium methosulphate.

Group 7

1367. — 2:7 - Bis - (2 - dihydroglyoxalinyl) - 9 - phenylphenanthridine trihydrochloride dihydrate.

1393. — 2:7 - Bis - (2 - dihydroglyoxalinyl) - 10-methyl-9-phenylphenanthridinium chloride dihydrochloride tetrahydrate.

742. — 2:7-Bis-(N-phenylamidino)-9-phenylphenanthridine monohydrate.

Group 8—Diazapyrenes (Fairfull, Peak, Short, and Watkins, 1952).

1015. — 2:7-Diphenyl-1:6-diazapyrene-1:6-bismethiodide dihydrate.

1428. — 2:7-Di-(4-aminophenyl)-1:6-diazapyrene-1:6-bis-methomethane-sulphonate.

1408. — 4:9-Diamino-2:7-diphenyl-1:6-diazapyrene-1:6-bis-methochloride.

The compounds or their salts were soluble at 1% (w/v) in water. An exception, No. 1343, was supplied as the glucoside, which was soluble.

Apart from the question of any correlation between structure and activity, the series offered an opportunity for the further assessment of the value of bacteriophage as a test virus for screening possible antivirus agents. A bacteriophage test is very convenient if one considers the phage as one particular test virus and if one is aware of the limitations of such tests.

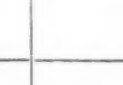
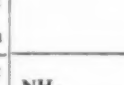
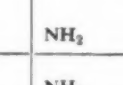
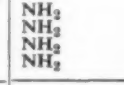
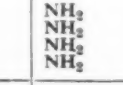
METHODS

Ps. Pyocyanea Phage.—The method used was described by Dickinson (1948) and modified by Chantrill *et al.* (1952). Tenfold dilutions of the compound under test were made in a synthetic medium and inoculated with host bacterium *Ps. pyocyanea* and phage Pb. After incubation for 24 hours the cultures were spotted on an indicator plate, previously sown with the sensitive host culture; this plate was then incubated for 24 hours. Provided that the host had grown in the dilution tube, an area of "no lysis" denoted that the phage had not grown in that tube. Results were expressed as:

$$\frac{\text{Log. titre phage inhibition}}{\text{Log. titre host inhibition}}$$

TABLE I

ANTIVIRUS ACTIVITY OF THE PHENANTHRIDINES

No	Structure of Compound: 2	7	9	10	*Ps. pyocyanea* Phage: Log. Phage Titre/Log. Host Titre	Anti-bacterial Action	Influenza A: Toxic Dose (micromol./egg)	Influenza A: Dilution of Toxic Dose Active (Reciprocal)	Rous Sarcoma (Chick Test)	Acute Toxicity LD50 in Mice (Subcutaneous mg./g.)
Group 1:										
1095			PhNH		3/3	0	>10	—	—	>0·5*
1279			PhNH	CH_3	4/4	+	—	—	—	>0·5
1280			$PhNCO_2C_2H_5$	CH_3	6/6	+	>20	0	—	>0·5
Group 2: Phenidium		NH_2	*p*-$NH_2C_6H_4$	CH_3	5/3	+++	10	0	0T	0·09
Group 3:										
481	NH_2	NH_2	Ph	CH_3	5/4	+++	5–10	?2	PT	0·07
1602	NH_2	NH_2	Ph	C_5H_{11}	5/4	+++	1·2	4	+T	0·1
1470	NH_2	NH_2	Ph	C_6H_{13}	6/4	+++	0·62	4	+T	0·07
1446	NH_2	NH_2	Ph	$CH_2CH{=}CH_2$	5/4	+++	2·5	2	—	0·14
Group 4:										
1390	NH_2	NH_2	*p*-$NH_2C_6H_4$	CH_3	5/3	++	10	?2	—	>0·2
1343	NH_2	NH_2	*p*-$NH_2C_6H_4$	(as glucoside)	4/3	++	>20	0	—	1·5
1662	NH_2	NH_2	*p*-$NO_2C_6H_4$	C_2H_5	6/3	+++	5–10	4	—	0·14
1601	NH_2	NH_2	*p*-$NO_2C_6H_4$	C_3H_7	4/<3	+++	5	2	—	0·28
Group 5:										
2202	NH_2	NH_2	CH_3	CH_3	5/3	+++	10	0	—	0·07
1098	NH_2	NH_2	C_4H_9		3/3	+	20	0	—	0·08
1120	NH_2	NH_2	C_4H_9	CH_3	5/3	+++	5	0	P	0·07
Group 6:										
1099	C_2H_5COONH	C_2H_5COONH	CH_3	CH_3	4/3	++	1·2	0	—	0·15
1097	C_2H_5COONH	C_2H_5COONH	C_4H_9	CH_3	5/3	+++	0·3	?2	0T	0·05
Group 7:										
1367			Ph		5/<3	+	2·5	4	PT	0·2
1393			Ph	CH_3	<3/<3	—	>2·5	0	—	0·04
742			Ph		4/4	0	5	0	—	>0·9
	1 / 2	4	6 / 7	9						
Group 8	see formula page 139									
1015	CH_3 / Ph		CH_3 / Ph		3/<3	+	0·62	0	PT	0·0025
1428	CH_3 / *p*-$NH_2C_6H_4$		CH_3 / *p*-$NH_2C_6H_4$		5/3	+	5	2–4	PT	0·04
1408	CH_3 / Ph	NH_2	CH_3 / Ph	NH_2	5/<3	+++	5	4	—	0·06

0=Inactive. P=Partial activity. T=Toxic. —=Not tested. ?=Activity not demonstrated in every test. *=I.P. figure, S.C. not available. +, ++, +++=Degree of activity (see Methods).

Influenza A Virus.—The methods described by Chantrill *et al.* (1952) were used. For *in ovo* tests the compound was introduced into the allantoic sac of 10-day-old fertile eggs; one hour later about 100 egg-infective doses of influenza A virus (PR8 strain) were injected by the same route. After 48 hours' incubation the allantoic fluid was tested for its ability to agglutinate fowl cells. Control tests for false haemagglutination and haemagglutination inhibition were always made. Fourfold dilutions of the compounds were tested, starting at M/20 and including a dose which was not toxic to the egg. Three eggs per dilution were usually tested, but any equivocal results were confirmed by further tests using twofold dilutions of the compounds. Results, expressed as a factor, referred to the dose in micromols. which was toxic to the chick embryo; thus a factor of 4 denoted that the compound was active at one-quarter the toxic dose.

Nos. 481 and 1367 were tested against mouse-adapted influenza A virus *in vivo*. Mice were infected intranasally with 100 infective doses of virus, the drugs being given twice daily subcutaneously, starting the day before infection. Survivors were killed seven days after infection and the lungs examined.

General Antibacterial Action.—The simple ditch-plate technique was considered sufficiently accurate for this series of compounds. A ditch containing 1/1,000 of the compound was made in a 5% blood-agar plate and the following organisms streaked across it: (1) *Streptococcus haemolyticus* (Richards); (2) *Staphylococcus aureus,* 663; (3) *Proteus vulgaris,* N.C.T.C. 5887; (4) *Pseudomonas pyocyanea.* 10; (5) *Salmonella aertryke*; (6) *Shigella shigae*; (7) *Shigella sonnei*; (8) *Corynebacterium pyogenes*; (9) *Streptococcus agalactiae,* N.C.T.C. 6175; (10) *Haemophilus pertussis*; (11) *Brucella abortus,* N.C.T.C. 6059. After 24 hours' incubation at 37° C. the width of the zone of inhibition was measured in mm.

Zone diameters were summarized for publication as: 0 (no inhibition against any organisms), + (slight inhibition, usually on the ditch only, against Gram-positive organisms and Nos. 8–11), ++ (inhibition of up to 10 mm. against Gram-positive organisms and Nos. 8–11), +++ (inhibition of 10–20 mm. against Gram-positive organisms and Nos. 8–11, with a slight inhibition against the Gram-negative organisms Nos. 3–7).

Rous Sarcoma Virus.—The method using young chicks was described by Dickinson and Thompson (1952). Both virus and drug solutions were inoculated subcutaneously on to the parietal peritoneal membrane, the first drug dose being given 24 hours after the virus. After eight daily doses of drug, the chicks were killed and the membranes examined visually for the presence of lesions produced by the virus. Assessments of 0–3 were given and compared with untreated controls; results were only considered significant if "p" <0.05. Chicks were weighed daily to see whether the drugs were toxic; sometimes toxic symptoms occurred—e.g., haemorrhagic membranes.

RESULTS

Results for all tests appear in Table I; the subcutaneous LD50 for mice is included, by courtesy of Dr. M. R. Gurd, as a measure of the acute toxicity of the compounds. No compound was active against influenza A virus at less than a quarter of the dose toxic to the chick embryo. One of the most promising, 1367, was inactive in mice at the maximum tolerated dose (Table II). Furthermore, several of the active compounds, 1367, 1408, 1470, 1602, were inactive against larger inocula (10,000 E.I.D.) of influenza virus. The results of the Rous sarcoma virus tests (Table III) also indi-

TABLE II

ACTION OF 481 AND 1367 AGAINST INFLUENZA A VIRUS IN MICE

Dosage mg./g./Day	Deaths Due to Toxicity (Within 2 Days)	Deaths Due to Virus	Infected Lungs of Survivors
481:			
0·02 ..	0/27	13/27	11/14
0·05 ..	2/12	Test discontinued*	—
0·1 ..	7/12	,, ,,	—
1367:			
0·02 ..	0/12	5/12	7/7
0·05 ..	1/9	6/9	1/2
0·1 ..	1st dose toxic	Test discontinued*	—
Controls ..	0/12	7/12	5/5

* This dose was toxic, many mice dying within two days and others obviously suffering from toxic effects.

TABLE III

ACTION OF PHENANTHRIDINES AGAINST ROUS SARCOMA VIRUS IN CHICKS (SUBCUTANEOUS ROUTE OF INFECTION)

Treatment commenced 24 hr. after infection

Compound	Daily Dose by Subcutaneous Route for 8 Days	No. of Chicks	Average Gain in Weight: Treated/Control*	Non-specific Deaths or Toxic Deaths	Parietal Peritoneum Examined 9–11 Days After Infection	
					Individual Assessments (Maximum 3)	Average Assessment
1st test:						
1602 ..	0·3 μg./g.	5	$0·59_{11}$ T	0	0, 0·5, 0, 1, 1	0·5†
1470 ..	0·3 μg./g.	5	$0·68_{11}$ T	0	0·5, 0, 0, 0, 0	0·1†
1097 ..	0·3 μg./g.	5	$0·55_{11}$ T	0	1, 3, 3, 3, 3	2·6
Controls	—	5	—	1 N S	3, 3, 3, 3	3·0
2nd test	(dose 0·002 micromol./g. body wt.)					
Phenidium	0·6 μg./g.	6	$0·60_{10}$ T	1 T	2·5, 3, 1·5, 1·5, 3	2·3
481 ..	0·6 μg./g.	6	$0·65_{10}$ T	1 T	1, 1, 2·5, 1, 1·5	1·4†
1470 ..	0·6 μg./g.	6	$0·79_{10}$ T	0	0, 0, 0, 0, 0, 0	0†
1120 ..	0·5 μg./g.	6	$0·89_{10}$	0	1·5, 1·5, 1, 1·5, 1, 1·5	1·3†
1367 ..	0·8 μg./g.	6	0·72 T	1 N.S.	1, 2, 2·5, 1, 2	1·7‡
1015 ..	1·0 μg./g.	6	0·79 T	0	1·5, 2, 2, 1·5, 1·5, 0·5	1·5†
1428 ..	0·9 μg./g.	6	0·73 T	0	0·5, 1·5, 2, 1, 1·5, 1	1·25†
Controls	—	12	—	0	3, 2, 2, 3, 2, 1·5, 2, 2·5, 2, 3, 1, 2	2·2

* Suffix to ratio refers to the period of days over which the average weight gain was calculated. † "p" = <0·001. ‡ "p" = <0·05. T = Toxic.

cate that the compounds are active only at a dose toxic to the host. No. 1367 had previously been reported as inactive at the maximum dose which was not toxic to the chick (Dickinson and Thompson, 1952).

The high ratios in the anti-phage tests were probably due to the relative resistance of the bacterial host, *Ps. pyocyanea*. This organism, like the other Gram-negative bacilli, was much less susceptible than the Gram-positive organisms. Three compounds, 1095, 1393, and 1098, were inactive against the host at the lowest dilution (1/1,000) tested and also inactive against phage. Three other compounds, 1279, 1280, and 742, although showing activity against the host, were inactive against phage at the highest drug concentration which permitted host growth.

Discussion

Little can be deduced regarding the correlation of structure and activity. Brownlee *et al.* (1950) found that the phenanthridines possess marked antibacterial activity, but that their high toxicity precludes their use in bacterial infections. Toxicity to the host probably accounts for the inactivity of 1367 in mice; even in eggs the therapeutic ratio is only 4. Egg toxicity figures vary, apparently quite independently of other activities, from 0.3 to >20 micromol./egg. The compounds which are not toxic to eggs are inactive against influenza, but compounds markedly toxic to eggs are not necessarily active.

No compound found active against influenza is inactive against phage, but some compounds of equal activity against phage are inactive against influenza. There is no quantitative relationship, but the bacterial host is unlikely to respond similarly to the fertile egg. One can only say that the antiviral activity of this class of compounds was detected in the first instance by the phage screening test. It would have been "missed" by the usual egg tests, where it is not usual to test such narrowly spaced dilutions as are necessary to detect the anti-influenza action of the phenanthridines.

Although of no obvious chemotherapeutic interest the phenanthridines provide a useful tool for investigations into bacteriophage production. No. 1367 is being used for such work; it behaves anomalously in that it would be expected to be less active than 1393, rather than more, because the ring nitrogen is not quaternized.

Summary

1. Twenty-three phenanthridines and related compounds were tested against influenza A virus in eggs, against Pb phage of *Ps. pyocyanea*, and against a range of bacteria.

2. Many compounds possessed marked anti-phage action, up to 1,000 times that on the bacterial host. The host, *Ps. pyocyanea*, and other Gram-negative bacilli were much less susceptible than the Gram-positive bacteria.

3. Several of the compounds active against phage possessed slight anti-influenza activity in eggs; the most promising of these compounds was, however, inactive in mice.

4. Seven of nine compounds had a suppressive action against Rous sarcoma virus in chicks, but only at a toxic dose.

The authors wish to acknowledge the interest of Mr. C. E. Coulthard and the late Sir Jack Drummond. They thank Drs. W. F. Short, T. I. Watkins, and A. E. S. Fairfull of the Chemistry Division for the compounds, and Dr. M. R. Gurd of the Pharmacology Division for the mouse toxicity figures.

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THE ACTION OF 2:7-BIS(2′-DIHYDROGLYOXALINYL)-9-PHENYLPHENANTHRIDINE ON A BACTERIOPHAGE OF *PSEUDOMONAS PYOCYANEA*

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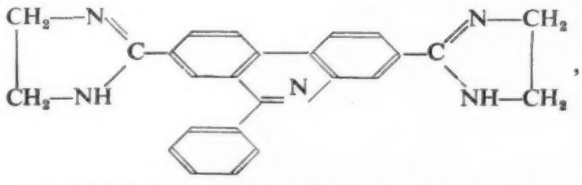
During investigations into virus chemotherapy, a number of phenanthridines were found active against bacteriophage Pb of *Pseudomonas pyocyanea* (Dickinson, Chantrill, Inkley, and Thompson, 1953). One of these, No. 1367, 2 : 7-bis(2′-dihydroglyoxalinyl)-9-phenylphenanthridine trihydrochloride dihydrate,



was chosen for more detailed investigation. Some preliminary results were reported by Dickinson and Codd (1952). They found that 1367 prevented multiplication of phage Pb at 0.01 mg./ml., but allowed host growth at 1 mg./ml. They considered the activity to be too great to be due to the slight action of 1367 on free phage Pb. The work reported here extends their study to: (i) the action of 1367 on the adsorption of phage Pb on its host, (ii) the effect on intracellular phage when No. 1367 is added at different times during the latent period, and (iii) antagonists of the drug.

METHODS

Strain C10 of *Ps. pyocyanea* (Dickinson, 1948) was used as host for phage Pb. It was maintained by daily subcultures in nutrient broth. Monthly subcultures were made from nutrient agar slopes stored at 5° C.

The stock of Pb phage was prepared by inoculating 100 ml. nutrient broth with 1 ml. 24 hr. broth culture of strain C10 and 10^4 phage particles. After incubating 24 hr. at 37° C. it was filtered through a gradacol membrane of porosity 1 μ. The final titre was 4×10^9/ml. For use it was diluted 10^{-2} in Ringer's solution and stored at 5° C.

The medium of Dickinson (1948) was used; it was termed medium B. It consisted of NaCl, 5 g.; $(NH_4)_2HPO_4$, 1 g.; KH_2PO_4, 1 g.; $MgSO_4.7H_2O$, 0.2 g.; lactic acid, 2.4 g.; casein hydrolysate (vitamin free), 1 g.; water, 1 litre. The *p*H was adjusted to 7.4 with NaOH and the medium sterilized by autoclaving at 15 lb./sq. in. pressure for 15 min. The final *p*H was 7.2.

Titration of Bacteriophage.—1 ml. of a 24 hr. broth culture of strain C10 was added to 1 ml. phage in a tube, followed by 3 ml. nutrient agar. After mixing the contents the tubes were "sloped" and incubated at 37° C. Counts were made after 18 hr. They were always approximately one-quarter of those made on plates. The reduction was probably due to the greater depth of agar in the tubes, as plaques were only observed on the agar surface.

Phage Multiplication.—The method for investigating phage multiplication was essentially that of Dickinson and Codd (1952). A 24 hr. culture of strain C10 on medium B was mixed with phage to give 10^8 viable bacteria and 10^6 phage particles/ml. After time for adsorption (5 min. when adsorption was in medium B, 10 min. when in Ringer's solution), the mixture was diluted in medium B and distributed in tubes to give 20–40 plaque-forming particles/tube. With this number, sufficient adsorbed phage was present to give fairly uniform counts in different tubes after a burst had occurred. This reduced the number of replicate tubes needed to give significant results. The action of No. 1367 could be investigated by adding it to the adsorption medium or to the tubes after adsorption and dilution.

This method was originally designed for latent period experiments and had not the sensitivity of the classical system of 1 phage/1 bacterium/tube of Ellis and Delbrück (1939). In particular it could not distinguish between reduction in phage yield due to reduced burst size and that due to fewer bursts. Such distinction, though, was not important in this work, and the disadvantage was greatly outweighed by economy in apparatus and labour.

Except for latent period measurements counts were made at only two times—once during the latent period and once after the rise period had ended. All times were measured from the moment when phage started to grow on the host, i.e., from mixing phage and host when adsorption was in medium B, and from dilution into medium B when adsorption was in Ringer's solution.

Results

Action on the Host.—Dickinson and Codd (1952) could find no action of No. 1367 on the growth rate of young (<12 hr.) cultures of strain C10 when tested at a concentration of 0.1 mg./ml. No attempt was made to repeat this work in detail.

Action on Phage

Active Concentration of No. 1367.—A preliminary test was made by the serial dilution method of Chantrill, Coulthard, Dickinson, Inkley, Morris, and Pyle (1952). No. 1367 allowed host growth at 1 mg./ml. and stopped growth of phage Pb at 0.005 mg./ml. in synthetic medium. A latent period experiment was carried out with No. 1367 present throughout at concentrations from 0.2 mg./ml. to 0.025 mg./ml. At 0.1 mg./ml. there was neither phage multiplication nor much phage loss (Fig. 1).

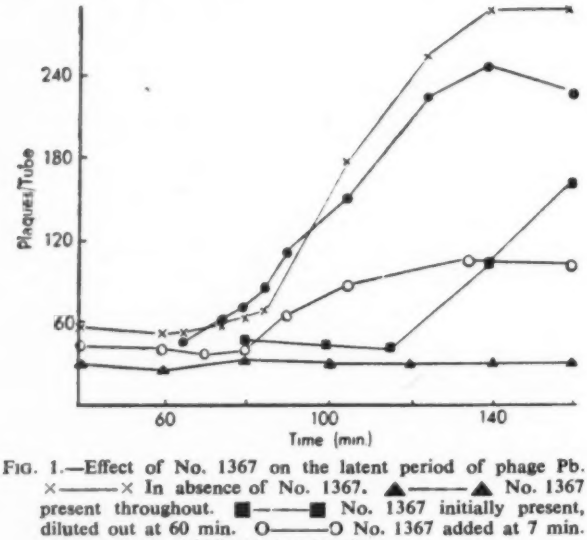


FIG. 1.—Effect of No. 1367 on the latent period of phage Pb. ×———× In absence of No. 1367. ▲———▲ No. 1367 present throughout. ■———■ No. 1367 initially present, diluted out at 60 min. O———O No. 1367 added at 7 min. ●———● No. 1367 added at 60 min.

For this concentration the fivefold dilution in nutrient broth and agar when sloping the tubes was sufficient to allow plaques to appear. This concentration was used for the rest of the work reported here.

Action on Free Phage.—An exposure of 2½ hr. to 0.1 mg./ml. of No. 1367 reduced the phage count to about two-thirds the initial level (Table I). This agreed with the results of Dickinson and Codd (1952) when allowance was made for the 24 hr. exposure used by them. If No. 1367 was absent the phage count increased fivefold in 2½ hr. in a latent period experiment (Fig. 1), so the total suppression of multiplication could not be due solely to action on free phage.

TABLE I

ACTION OF NO. 1367 AND GUANOSINE ON FREE Pb PHAGE

Compound	Concentration (mg./ml.)	Phage Count at 0 hr.	Phage Count at 2½ hr.
—	—	134, 154, 169	133, 114, 162
Guanosine ..	0·5	205, 221, 229	173, 173, 173
No. 1367 ..	0·1	116, 139, 159	79, 79, 95

Action on Adsorption (Table II).—0.1 mg./ml. of No. 1367 was present during adsorption. After adsorption it was diluted to one-thousandth of the minimum active concentration, before distribution in tubes, to prevent any further action on the phage. The phage yield at 2½ hr. was reduced by this treatment, but a burst still occurred. Consequently, though the reduction might be due to a direct action on adsorption, there must have been some action on intracellular phage. The control, in which the drug was present only after dilution, gave very variable results in different experiments. Its behaviour is discussed in detail later.

TABLE II

ACTION OF NO. 1367 ON THE ADSORPTION OF Pb PHAGE ON TO ITS HOST

Concentration of No. 1367, 0·1 mg./ml.

No. 1367 During Adsorption	No. 1367 After Adsorption	Count at 20 min.	Count at 2½ hr.
−	−	37, 37, 29	286, 290, 237
+	−	24, 32, 27	191, 187, 183
+	+	22, 22, 27	11, 13, 9
−	+	27, 33, 20	109, 130, 123

Attempts at Antagonism.—The following nucleic acid derivatives were used in attempts to antagonize No. 1367 in one-step growth experiments:

Adenine, guanine, thymine, uracil;
adenosine, guanosine, cytidine;
adenylic acid, guanylic acid, cytidilic acid;
yeast and thymus nucleic acids (B.D.H.).

The nucleosides and nucleotides were ribose derivatives. The compounds were prepared as 1:200 solutions or suspensions in medium B, *p*H 7. Each was tested at a concentration of 0.5 mg./ml. by addition to adsorption medium, medium after dilution, or both, in presence and absence of No. 1367. The nucleic acids were also tested at a concentration of 2 mg./ml., as they (but not the other compounds) antagonized No. 1367 in a serial dilution test at this concentration (Dickinson, personal communication).

Only yeast and thymus nucleic acids at 2 mg./ml. antagonized the phenanthridine ($p<0.05$), and then

only when nucleic acid and phenanthridine were present together. Thymus nucleic acid (Table III) was much more effective than yeast nucleic acid. Under these conditions yeast nucleic acid gave a precipitate with No. 1367. None was observed with thymus nucleic acid, but this may have been due to masking by the initially turbid solution. In absence of No. 1367 thymus nucleic acid reduced phage yield slightly (Table III). No further work has been done on this.

TABLE III

EFFECT OF NUCLEIC ACIDS AND NO. 1367 ON THE YIELD OF Pb PHAGE

Concentration of No. 1367, 0·1 mg./ml.; of nucleic acid, 2 mg./ml.

Additions to Adsorption Medium	Additions to Dilution Medium	Phage Count at	
		20 min.	2½ hr.
—	—	37, 36, 38	148, 158, 161
—	Thymus nucleic acid	39, 32, 24	128, —, 135
No. 1367 ..	—	25, 22, 25	171, 132, 121
,, ,, ..	Thymus nucleic acid	18, 17, 19	97, 98, 108
,, ,, ..	No. 1367	26, 20, 27	17, 21, 22
,, ,, ..	No. 1367 and thymus nucleic acid	37, 35, 20	112, 97, 106
—	—	37, 41, 47	314, 325, 333
—	Yeast nucleic acid	37, 31, 33	295, 292, 248
No. 1367 ..	—	42, 52, 51	228, 248, 218
,, ,, ..	Yeast nucleic acid	45, 38, 41	190, 213, 245
,, ,, ..	No. 1367	21, 37, 18	23, 27, 18
,, ,, ..	No. 1367 and yeast nucleic acid	29, 32, 21	65, 66, 48

Guanosine and adenosine both reduced phage yield when present after dilution if No. 1367 was absent, guanosine showing the effect more clearly. The effect was more obvious after adsorption in No. 1367, but also occurred after adsorption in its absence (Table IV). Under the conditions used guanosine had no effect on free phage Pb (Table I). The action of adenosine was not investigated.

TABLE IV

EFFECT OF GUANOSINE AND NO. 1367 ON THE YIELD OF Pb PHAGE

Concentration of No. 1367, 0·1 mg./ml.; of guanosine, 0·5 mg./ml.

Additions to Adsorption Medium	Additions to Dilution Medium	Count at	
		20 min.	2½ hr.
—	—	49, 46, 37	384, 347, 307
—	Guanosine	56, 49, 38	323, 305, 356
No. 1367 ..	—	49, 49, 50	213, 241, 226
,, ,, ..	Guanosine	41, 48, 47	180, 188, 178
,, ,, ..	No. 1367	25, 25, 31	13, 23, 14
,, ,, ..	No. 1367 and guanosine	25, 32, 26	22, 17, 18

Action on Intracellular Phage.—In the above work medium B had been used as the adsorption medium. Phage particles adsorbed at the beginning of a 5 min. adsorption period would have undergone 5 min. development before No. 1367 was added after dilution; this would mask any action it had on the early stages of multiplication. To overcome this, adsorption was carried out with starved host cells suspended in Ringer's solution. (Benzer (1952) had previously shown that T_2 phage adsorbed on starved cells of *Escherichia coli B* underwent very little development.) To increase sensitivity a further modification was introduced. After adsorption the host-phage mixture was diluted 10^{-3} into medium B, giving ~4,000 particles/ml. No. 1367 was added to this. The mixture was diluted to 20–200 particles/ml. immediately before sloping. This treatment gave many more bursts/tube and so reduced variation due to differences in burst size and distribution of bursts among the tubes.

When 0.1 mg./ml. of No. 1367 was added less than 3 min. after the end of adsorption, there was no phage multiplication. When it was added between 3 min. and 12 min. phage yield increased rapidly with time of addition of the phenanthridine. From 12 min. to 80 min. the yield was fairly constant and considerably less than that of controls (Fig. 2).

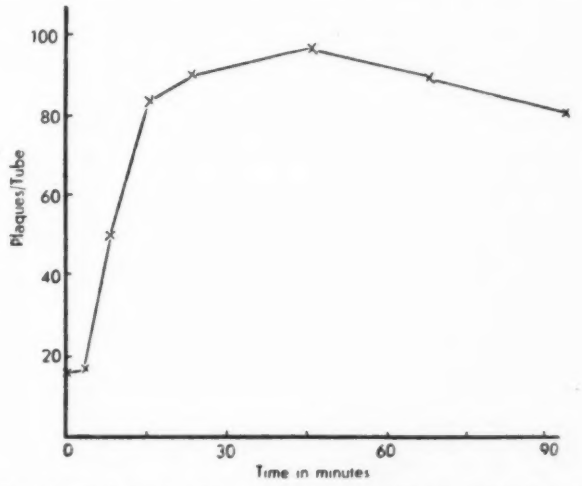


FIG. 2.—Variation in yield of Pb phage at 150 min. with time of addition of No. 1367. Initial count was 23/ml. Count at 150 min. in absence of No. 1367 was 120/ml.

Action on Latent Period.—When 0.1 mg./ml. of No. 1367 was added at 7 min. and then the latent period measured, no difference from controls was found (Fig. 1), so the effect of No. 1367 under these conditions was directly on phage multiplication. For addition between 12 min. and 80 min. this seemed unlikely. Premature lysis of infected cells, as had been found by Hotchin (1951) for some acridines on staphylococcus phage K, appeared more probable. This was confirmed (Fig. 1) for addition of No. 1367 at 60 min.

Effect of Diluting Out.—Adsorption was carried out in presence of 0.1 mg./ml. of No. 1367 and the host-phage mixture diluted into medium B + No. 1367. At 60 min. the drug was diluted out and then the latent period measured. It was about 50 min. longer than that of a control omitting the drug (Fig. 1).

Discussion

The principal action of No. 1367 clearly is exerted in the first three minutes after infection, though there is a less complete effect on later stages of development. Similar results (unpublished work) have also been found for T_1 and T_2 phages of *Esch. coli B*. Its action is very different from that of proflavine on the T phages of *Esch. coli B* (Foster, 1948), where only a very late stage in phage synthesis is inhibited. The results allow no definite conclusions on the mechanism of action of No. 1367. They suggest that it interferes with reorganization of the infecting phage rather than with the synthesis of new phage material.

The antagonism of No. 1367 by yeast and thymus nucleic acids is not considered to imply that No. 1367 acts by preventing nucleic acid synthesis. It seems more likely that they combine with the phenanthridine and lower its effective concentration below the limit of activity.

The reduction in yield of phage Pb by guanosine was unexpected, and no explanation is offered for it. It is perhaps interesting, though, that guanosine has recently been found to act on *Esch. coli B* as an antimutagen (Novick and Szilard, 1952).

Summary

1. 2:7-Bis(2′-dihydroglyoxalinyl)-9-phenylphenanthridine trihydrochloride (No. 1367) had little action on free phage Pb or on the adsorption of phage Pb on the host bacterium.

2. It prevented multiplication of phage Pb if added during the first three minutes of phage growth.

3. If added when the phage had been growing more than 3 min. the phage yield varied with time of addition.

4. The phenanthridine did not affect the latent period of phage Pb when added at 7 min. It reduced it slightly when added at 60 min.

5. When the phenanthridine was initially present and later diluted out, the latent period of phage Pb was increased.

6. Yeast and thymus nucleic acids antagonized the action of the phenanthridine in one-step growth experiments. The action of thymus nucleic acid was much the stronger. They acted only when nucleic acid and phenanthridine were together. Nucleotides, nucleosides, purines, and pyrimidines tested failed to antagonize it.

7. Guanosine and adenosine reduced the yield of phage Pb in one-step growth experiments. Guanosine had little action on free phage Pb.

The author is glad to express the debt he owes to Dr. Löis Dickinson for her advice and criticisms throughout this work, and to acknowledge the interest taken by the late Sir Jack Drummond, F.R.S., and Mr. C. E. Coulthard in it. He wishes to thank Miss Ann Wheater for technical assistance.

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THE NICOTINE-LIKE ACTIONS OF THE 3-BROMO- AND 3 : 5-DIBROMO-PHENYL ETHERS OF CHOLINE (MBF AND DBF)

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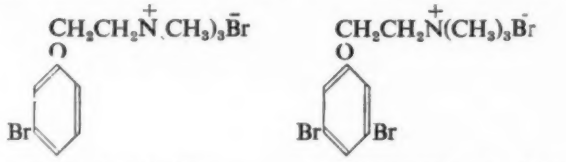
The 3-bromo- and 3 : 5-dibromo-phenyl ethers of choline were first synthesized by Hey (1952); their structures are shown in Fig. 1, and they will be referred to as MBF and DBF respectively. Hey



3-Bromophenyl ether of choline bromide (MBF)

3 : 5-Dibromophenyl ether of choline bromide (DBF)

FIG. 1

tested these new drugs for nicotinic activity by examining their effect on the blood pressure of atropinized cats. By this test the potency of MBF and DBF, on a molar basis, was 2–3 times that of the unsubstituted phenyl ether of choline and 4–5 times that of nicotine. The rise in blood pressure produced by these new derivatives persisted after ligation of the suprarenals, and was abolished by known ganglion-blocking agents. The pressor action found by Hey therefore implies an ability of MBF and DBF to excite autonomic ganglia. In the present series of experiments on the perfused superior cervical ganglion of cats we have confirmed the fact that these compounds are endowed with a powerful ganglionic action. Additional nicotine-like properties on a number of other tissues, including skeletal muscle, are described in this paper. For instance, it is shown that MBF and DBF elicit pilomotor axon reflexes in the human skin, a type of cutaneous reaction due to stimulation of axonic endings, and first noted after intradermal injection of nicotine by Coon and Rothman (1940). This "axonic" action of MBF and DBF (and also that of nicotine) is blocked by hexamethonium. The existence of an action of this type on a set of autonomic nerve-endings gives rise to the theoretical possibility that compounds endowed with nicotinic activity, such as these and possibly others, may exert an effect at both ends of autonomic neurones, a ganglionic action on the cell bodies and an "axonic" action at their terminals.

The concluding section of this paper deals with the action of MBF and DBF on isolated mammalian intestines. Our observations have been confined to the effect on the longitudinal muscle layer, principally in rabbits. It is shown that MBF and DBF elicit contractions which are blocked by hexamethonium and reversed by botulinum toxin. Thus the motor action of MBF resembles that of nicotine in these and other respects, and can likewise be attributed to a stimulation of the motor neurones in the enteric plexus. There is no evidence at present whether this "indirect" action is purely ganglionic or whether it also includes an "axonic" component.

METHODS

Technical details of the various types of preparation used for these experiments can be found in an earlier paper (Ambache, 1949). The following alterations need to be mentioned.

Frog's Rectus Abdominis.—The bath fluid was aerated.

Avian Iris.—Pigeons were used instead of hens. They were anaesthetized with 6–12 mg. of pentobarbitone sodium (nembutal) intramuscularly and with 1–2 drops of 1% amethocaine HCl instilled into the conjunctival sac. MBF or DBF was injected into the anterior chamber in a volume of 0.01–0.02 ml.

Ganglion Perfusion.—Cats were anaesthetized with 38 mg./kg. of pentobarbitone sodium intraperitoneally. The method for the perfusion of the superior cervical ganglion was as before except that (*a*) the use of heparin was omitted, (*b*) the glucose content of the Locke's solution for perfusion was doubled to

2 g./l., and (*c*) the perfusion fluid was warmed, by the animal's own body-heat, during its transit through a length of transparent "Portex" tubing inserted into the stomach and emerging from a lateral incision in the oesophagus at the root of the neck, where connection was made to the perfusion cannula. We are indebted to Professor F. C. MacIntosh for this suggestion. Contractions of the nictitating membrane were recorded with an isometric lever at a basal tension of 10 g.

Pilomotor Reaction.—MBF and DBF were injected intradermally in several human subjects in a volume of 0.05 ml. of 0.9% NaCl.

Isolated Intestine.—13 adult rabbits, 4 guinea-pigs, and 2 kittens were killed by a blow on the back of the neck. A 4–5 cm. portion of ileum was suspended, after emptying its lumen, in a 10-ml. organ-bath in Mg-free Tyrode's solution containing 1.1% $NaHCO_3$, and oxygenated with 95% O_2 and 5% CO_2 (for guinea-pig gut pure O_2 was used). The contractions of the longitudinal muscle were recorded with a frontal writing lever which had a fivefold magnification.

In five other experiments on normal rabbits the required segment of ileum was excised from the live animal under ether anaesthesia. The initial presence of ether in these preparations did not appear to affect the results in any noticeable way. Four other preparations were taken, also during ether anaesthesia, from rabbits in which the anterior mesenteric and both coeliac ganglia had been removed at a previous operation for another purpose. As there did not appear to be any noticeable difference in the pharmacological reactivity of these rabbit intestinal preparations from the three different sources mentioned, the results obtained on them will be presented together without further sub-classification.

Experiments with Botulinum Toxin.—The technique of injecting botulinum toxin subperitoneally into an intestinal segment has been described previously (Ambache, 1951). An important modification in procedure in the present experiments has been that, during the initial ether anaesthesia but before the injection of toxin, a 5–10 cm. segment of ileum was excised between ligatures; the middle 5 cm. of this was suspended at once in an organ-bath for control tests with drugs. An adjoining segment of gut, 10 cm. in length and situated 5–10 cm. above or below the site of excision, was then injected with 2–4.6 mg. of toxin A_2. The rabbits were killed by an injection of air into the marginal ear vein 1½–4½ hours later, and the injected segment was removed for pharmacological examination in the organ-bath. Thus each experiment was conducted in duplicate on two preparations from the same animal: the first a "control," the other treated with botulinum toxin. The two preparations were made as nearly equal in length as possible (4–5 cm. usually), and the same lever was used for both.

Drugs.—The bromide salts of MBF and DBF were obtained through the courtesy of Dr. P. Hey. All drug doses given in the text refer to the salts: nicotine as the hydrogen tartrate, hexamethonium as the bromide, *d*-tubocurarine as the chloride, atropine as the sulphate, muscarine as the chloride, etc.

Results

Nicotine-like Actions of MBF and DBF

Skeletal Muscle.—Both compounds are able to produce contractions of the frog's rectus abdominis muscle in the absence of eserine. MBF appears to be about twice as active as DBF (Fig. 2); *d*-tubocurarine (10^{-5}) antagonizes these effects, but the antagonism is reversible.

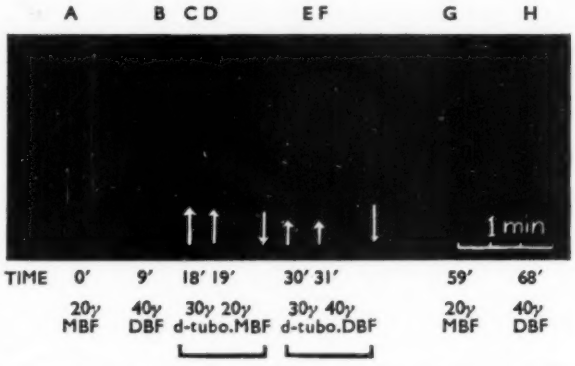


FIG. 2.—Frog's rectus abdominis in a 3 ml. bath. Actions of MBF and DBF, and reversible antagonism by *d*-tubocurarine. The minutes below refer to the time of administration of each drug. Drum stopped between tests. At A, D, and G, 20 μg. of MBF. At B, F, and H, 40 μg. of DBF. At C and E, 30 μg. of *d*-tubocurarine.

The skeletal muscle in the pigeon's iris also responds to these compounds, and 1–2 μg. of either drug, injected into the anterior chamber, promptly constricts the pupil (Fig. 3).

Ganglia.—From his analysis of the nicotine-like effect of these compounds on the cat's blood pressure, Hey (1952) inferred that MBF and DBF were capable of exciting autonomic ganglion-cells. In unpublished experiments Dr. Hey and his colleagues (personal communication) have also

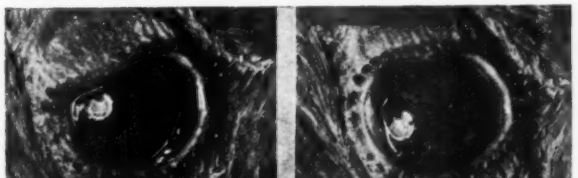


FIG. 3.—Pigeon's iris (*a*) before and (*b*) immediately after injection, into the anterior chamber, of 1.5 μg. of MBF in 0.15 ml. of 0.9% NaCl. Photographs taken with 0.2 msec. flashes.

observed a stimulation of the cat's superior cervical ganglion with these compounds. We have confirmed Hey's conclusions in the present series of ganglionic perfusions. Furthermore, our results show that, like all other substances endowed with nicotinic activity, MBF and DBF in larger doses produce a ganglion-cell paralysis which results in an arrest of synaptic transmission. In seven experiments we have obtained stimulation with doses of 0.05–0.1 μg. of MBF and 0.05–0.2 μg. of DBF. Block occurred after doses of 0.4–10 μg. of MBF or 0.05–1 μg. of DBF, and its intensity appeared to be proportional to the dose.

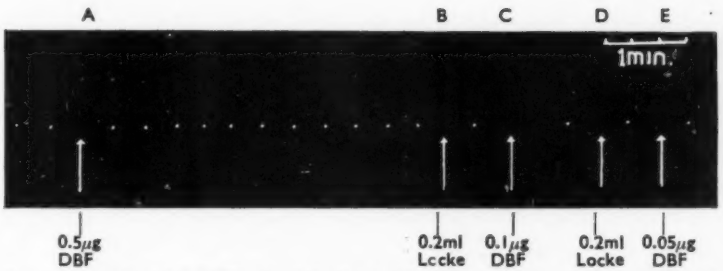


FIG. 5.—Continuation of Fig. 4. Injections of DBF: at A, 0.5 μg.; C, 0.1 μg.; and E, 0.05 μg. At B and D, 0.2 ml. of glucose-free Locke's solution. Following the stimulant action of DBF at A, there is complete block of the response to preganglionic stimulation for 3 min. and slow recovery during the next 7 min.

In a typical experiment injections of 1 μg. of MBF elicited effects which were nearly equal to those of maximal preganglionic stimulation at 20 cycles/sec. applied for 5 sec. The latency of the MBF responses was 2.5–3 sec. from the moment of injection. In order to detect the presence of block, "test" maximal preganglionic stimuli were delivered at minute intervals throughout the experiment. The first sign of block appeared after the second dose of 1 μg. of MBF. It was pronounced after 1.5 μg. and was complete for a period of 1 min. after 2 μg., and for 3 min. after 5 μg. It took 6–7 min. for transmission to recover fully after the 2 and 5 μg. doses of MBF, and also after 1 μg. of DBF.

In another experiment it was found that, during the phase of total block following injections of 1–2 μg. of MBF, maximal contractions of the nictitating membrane could still be elicited by postganglionic stimulation. This observation showed that the ineffectiveness of preganglionic stimuli was due to a block within the ganglion.

The block produced by 2 μg. of MBF and by 0.5 μg. of DBF, and the time-course of subsequent recovery, are shown in Figs. 4 and 5. In this experiment the response to 0.2 μg. of MBF was equated with that to 2 μg. of nicotine. As shown in Fig. 4, 0.5 and 1 μg. of MBF produced effects greater than that of a 5 sec. period of maximal preganglionic stimulation at 20 cycles/sec. Later on (not shown in Fig. 4) the response to 1 μg. of MBF was reversibly abolished by a 250 μg. dose of hexamethonium.

The last two experiments in this series were performed on "denervated" ganglia, the preganglionic nerve supply of which had been cut 9–15 weeks previously. The first of these ganglia responded to 0.5 μg. of MBF. In the second, maximal responses were obtained with 0.1 μg. of MBF; these were equated with 1 μg. of nicotine and were blocked by 500 μg. of hexamethonium or by 200 μg. of *d*-tubocurarine administered 1 min. previously. The ganglion-cell-paralysing properties of MBF were also evident in this experiment, as shown by the observation that the normal response to 5 μg. of acetylcholine was blocked 2 min. after a dose of 0.4 μg. of MBF, but recovered within 5 min. of that dose.

Pilomotor Nerve-endings.—In 1940 and 1941 Coon and Rothman discovered that a number of drugs possessing nicotinic activity could, on intradermal injection into the human skin, initiate axon reflexes in the branching terminals of several categories of autonomic nerve-fibres innervating structures in the skin. The localized pilomotor, sudomotor, and vasoconstrictor responses which they described provided the first examples of nicotine-induced axon reflexes at adrenergic and cholinergic motor nerve-endings respectively. In the introduction we have referred to this type of action as

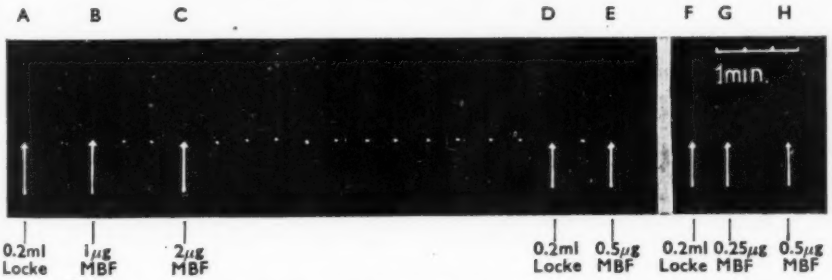


FIG. 4.—Perfusion of cat's superior cervical ganglion. The white dots indicate responses of the nictitating membrane (contraction upwards) to maximal preganglionic stimulation (20 cycles/sec. applied for 5 sec.). At A, D, and F, control injections of 0.2 ml. glucose-free Locke's solution. MBF injections: at B, 1 μg.; C, 2 μg.; E and H, 0.5 μg.; and G, 0.25 μg. Following the stimulant action of 2 μg. at C, there is complete block of the response to preganglionic stimulation for 1 min. and slow recovery during the next 9 min.

"axonic." Brown and Gray (1948) have shown, further, that nicotine can excite yet another kind of nerve-ending in the skin, namely, those subserving the sensation of touch. Later Douglas and Gray (1953) found that this action of nicotine, which may perhaps also be considered as "axonic," was abolished by hexamethonium. Thus it is clear that various types of nerve-ending may possess pharmacological properties hitherto considered as characteristic of autonomic ganglion cells. This fact is perhaps less surprising than would appear at first, since even in the superior cervical ganglion preganglionic fibres may come into synaptic, and therefore possibly into humoral, relation not only with the cell bodies of ganglia but also (Ranson and Billingsley, 1918, Fig. 15) with dendrites, i.e., with a type of nerve-ending.

Coon and Rothman made the suggestion that the reactions which they described might serve as specific pharmacological tests for the detection of nicotinic activity. We have found the pilomotor reflex the easiest to observe, and have subjected the action of MBF and DBF to this single test by injecting them intradermally into four human

FIG. 6.—Pilomotor axon reflex elicited by an intradermal injection of 0.3 μg. of MBF in 0.05 ml. of 0.9% NaCl.

subjects. Both substances are active in concentrations of $3–6\times10^{-6}$ for MBF and 5×10^{-7} to 2×10^{-6} for DBF. The effect begins within a few seconds of the intradermal injection and fades away after ¾ to 1¼ min. As shown in Fig. 6, the reaction consists of a circular area of gooseflesh with characteristic "pseudopodia" due to the erection of isolated groups of satellite hair follicles outside the main area of the response, sometimes at a considerable distance from it. The initiation of this reflex, whether by MBF, DBF, or by nicotine (10^{-5}), is abolished by previous intradermal injection of hexamethonium (10^{-4} or 10^{-3}) into the same site. Blocking properties were observed with higher concentrations of MBF (10^{-4}), which abolished the effect of 10^{-5} of nicotine.

Intestinal Actions of MBF and DBF

With the exception of those experiments below which deal with guinea-pig and kitten gut, the results reported here were obtained on the longitudinal muscle of the rabbit ileum. Although the bulk of this work was carried out with MBF, DBF was used in a few trials. In six of seven experiments 1–15 μg. of DBF elicited contractions, whilst 10–45 μg. produced relaxation; in four of these preparations variable mixtures of contraction and inhibition, or the converse state of affairs where relaxation preceded contraction, were sometimes observed. Therefore, as the results with MBF appeared to be more consistently uniform, DBF was discarded for the purposes of the present investigation, which was then confined to MBF.

Stimulating Action.—Longitudinal muscle contractions were elicited in 22 experiments on different preparations by MBF in doses of 2–50 μg. (final concentration in the organ-bath: 2×10^{-7} to 5×10^{-6}); in two of these experiments relaxation was produced by higher doses of MBF, i.e., by 50 and 100 μg. A noticeable feature of the motor response to MBF, also seen with DBF, was the decrease in the magnitude of the contractions recorded as the dose was increased. This phenomenon is illustrated in Fig. 7; the diminution in the height of the MBF contraction occurred as the dose was increased from 10 μg. at D to 25 μg. at E; 62 min. later the effect of 40 μg. of MBF at I was even smaller. This phenomenon may be related to the onset of a nicotine-like "paralysis" which will be discussed below.

The potency of MBF was compared with that of nicotine in 11 of these experiments. Satisfactory matching between two doses, one of MBF and the other of nicotine, was possible on most of the preparations as, for example, in Fig. 11 B and C. In this particular instance the activity ratio of MBF to nicotine was unity; in the remaining preparations where such matching was possible the ratio varied between 0.5 and 2 (the ratio for DBF was, in two experiments, 2 and 2.5 respectively). But on a few occasions matching was made difficult both by variations in the MBF response and also by the fact that large contractions could be obtained in response to nicotine but not to MBF because of the onset, as the dose of MBF was increased, of the "paralytic" effect described above. "Paralysis" may also account for the fact that the MBF contraction is often not sustained at a plateau and

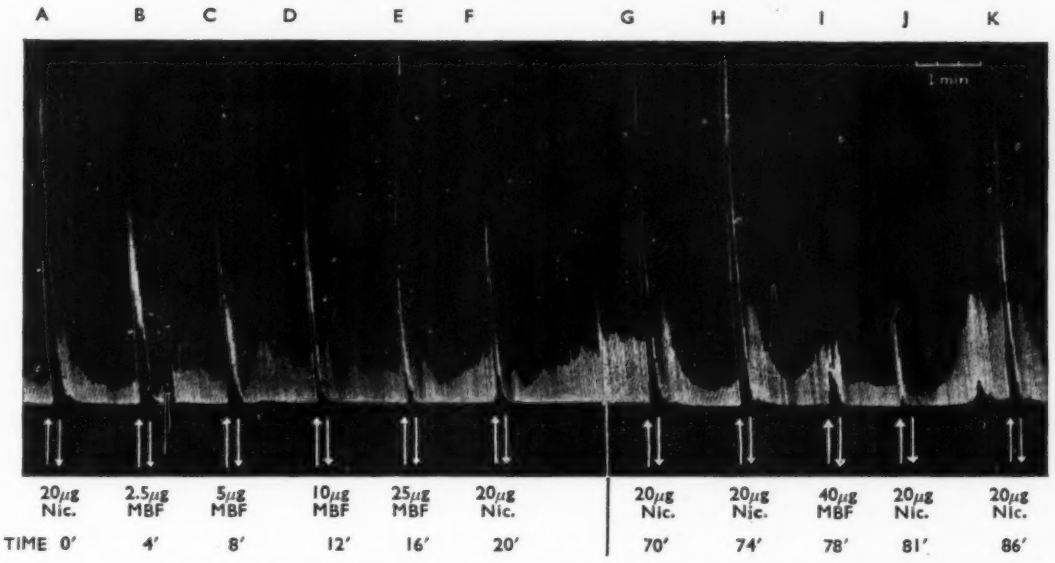


FIG. 7.—Rabbit, 2.2 kg. (pregnant). Ileum preparations 165 cm. below pylorus. All drugs left in the organ-bath (vol. 10 ml.) for 30 sec. only. Time in minutes at the bottom of the tracing. At A, F, G, H, J, and K: 20 μg. of nicotine. MBF at B, 2.5 μg.; C, 5 μg.; D, 10 μg.; E, 25 μg.; and I, 40 μg. Note the decrease in effect with the larger doses of MBF and the subsequent reversible depression of the response to nicotine (at F and J).

begins to subside before the drug is washed out of the organ-bath.

It has been shown by Feldberg (1951) that nicotine-induced intestinal contractions can be abolished by hexamethonium, an effect attributed at the time solely to the ganglion-blocking properties of hexamethonium. In the present series of experiments the motor response to MBF could likewise be abolished, or very greatly reduced, by hexamethonium in concentrations of $1–4 \times 10^{-4}$. Fig. 8 illustrates this antagonism; it shows the initial response to 20 μg. of MBF at A and B, its extinction at D in the presence of 4 mg. of hexamethonium, and lastly its recovery 3 min. later at E. The tracing also shows that the tone and rhythmic activity of the gut were affected by the presence of hexamethonium (at C). This was a usual finding, though the direction of the effect was unpredictable; it was sometimes inhibitory, as in this experiment, but in other preparations hexamethonium produced an augmentation of pendulum movements.

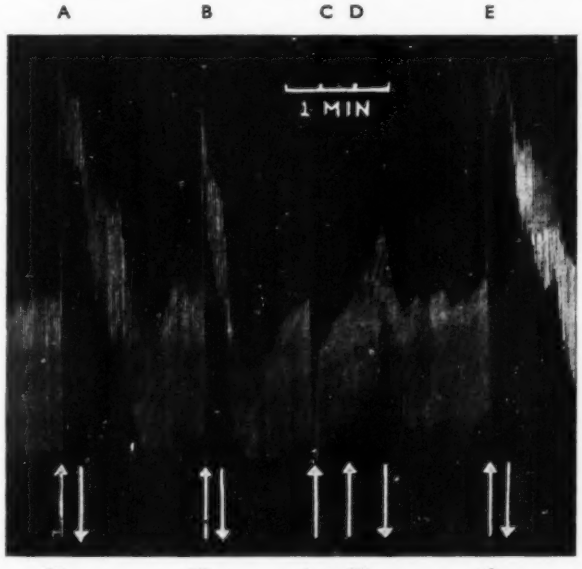


FIG. 8.—Reversible antagonism of MBF by hexamethonium in a rabbit's ileum preparation taken 35–40 cm. below pylorus. A, B, and E: 20 μg. MBF for 30 sec. C: 4 mg. hexamethonium producing a temporary inhibitory effect by itself and antagonizing, 60 sec. later, the effect of D, 20 μg. MBF for 60 sec.

Since, as shown above, MBF acts very much like nicotine at ganglia and nerve-endings, it would be reasonable to suppose that in the intestine, which incorporates a complex plexus of ganglion-cells and nerve-endings, the action of MBF would also resemble that of nicotine in many respects. The evidence presented so far supports this view. In particular the antagonism of MBF contractions by hexamethonium would seem to point to a localization of the motor action at least to the motor neurones within the gut. Further aspects of this "indirect" type of action are described below.

Blocking Action.—Several examples of the blocking properties of MBF have been given above, showing that in larger doses this compound renders nerve-cells and nerve-endings pharmacologically inexcitable. The experiments on the superior cervical ganglion show that this inexcitability may persist

for several minutes. It was to be expected that a similar block would occur in the gut, at least at the motor neurone level. The " paralytic " phenomena referred to in the preceding section are probably an expression of this type of block, as shown by the following considerations.

In the experiment of Fig. 7, and several other experiments of this type, the use of larger doses of MBF (and of DBF) was characterized not only by a diminution of the contraction to MBF itself but also by a subsequent depression of the response to a fixed dose of nicotine. As shown in Fig. 7, the response to a 20 μg. dose of nicotine was constant and repeatable even after a long period of time (see, for instance, A, G, and H). Four minutes after the 25 μg. dose of MBF at E the response to nicotine, at F, was reduced to less than half its original height at A. An interval of 50 min. was allowed for recovery before the next two test doses of nicotine at G and H, which elicited contractions of constant height again. The 40 μg. dose of MBF at I, although itself not causing any appreciable contraction, was followed 3 min. later at J by a reduction of the nicotine response to a quarter of its previous height and by recovery at K 5 min. later. It is necessary to stress the fact that this temporary inexcitability to nicotine is not due to an effect of the type described by Cantoni and Eastman (1946). These authors found that after a vigorous intestinal contraction the guinea-pig gut exhibits a depression of pharmacological responses; it is not clear as yet whether this effect is due to metabolic exhaustion after a powerful contractile effort, or to a state of true inexcitability. In the rabbit's intestine Beznák (1952) has shown that large contractions induced by acetylcholine are followed by temporary arrest of pendulum movements. However, the decreased response to nicotine in Fig. 7 J cannot be due to a phenomenon of this kind, since the administration of MBF at I was neither attended by contraction nor followed by an arrest of pendulum movements.

It is clear from these experiments that MBF can in small doses stimulate, and in excess depress, the particular structure or structures in the gut which normally respond to stimulating doses of nicotine. Although it would be surprising if the autonomic ganglia in the enteric plexus were insensitive to nicotine, it is still uncertain whether nicotine-sensitivity in the gut is restricted to the ganglion-cell bodies, since it is now known that nicotine is also capable of stimulating some types of nerve-endings. It is therefore theoretically possible that the " axonic " actions of nicotine and MBF may contribute partly to the effect of these drugs on the gut. In the absence of evidence on this point, it is perhaps advisable at present to think of the motor action of these drugs as being due to stimulation of the motor neurones in the gut, possibly at both ends of these neurones. Likewise the " paralytic " effect of larger doses of these drugs indicates a state of " block " in those neurones, not necessarily confined entirely to the ganglion-cell end.

Reversal by Botulinum Toxin.—The experiments with botulinum toxin provide further evidence of the " indirect " nature of the motor response to MBF. There are numerous examples of the ease and rapidity with which botulinum toxin is able to paralyse cholinergic nerves at their endings. And as it is commonly supposed that the motor neurones in the intestine are cholinergic, it should be possible, with this toxin, to put these neurones out of action by paralysing their endings. This opens the possibility of eliminating the contractile response to certain drugs whose principal effect on the gut is supposed to be due to stimulation of the motor neurones; it provides another means of examining to what extent the action of these drugs is " indirect." In fact, as was shown previously, when the rabbit's intestine is treated with toxin, the motor action of nicotine is eventually suppressed and then actually undergoes a complete reversal (Ambache, 1951). In the present experiments the motor effect of MBF has likewise been greatly reduced or even abolished by the toxin. In seven of nine experiments the extinction of the motor response to MBF proceeded far enough to allow a reversal to become apparent, as with nicotine. This may be looked upon merely as an unmasking, by the motor neurone paralysis, of an underlying inhibitory action of MBF, resembling the inhibitory action of nicotine which was discussed in the earlier paper. It is necessary to stress the fact that, in all the seven experiments in which reversal occurred, the inhibition was produced by doses of MBF which in the 22 normal preparations had always been motor. In order to emphasize the specificity of this reversal the following procedure was adopted in all but one of these experiments. As shown in Figs. 9 and 10, each experiment was begun by obtaining a match on the control segment between contractions produced by MBF (and sometimes by nicotine) on the one hand, and contractions produced by drugs possessing an intense direct (or " muscarinic ") action on the muscle fibres of the gut, such as 2268F, acetylcholine, or muscarine itself. After these matches were obtained on the control, the poisoned segment from the same animal was suspended in the organ-bath, and the previously matched doses of these various drugs were tried again. Whenever this was done it was

found that a given dose of MBF, which in the control segment of gut had elicited a contraction, would produce relaxation in the botulinized segment from the same animal. Moreover, the reversal produced by the toxin was restricted to the action of MBF (and of nicotine, Fig. 10 F). Thus, the motor effect of muscarine, of 2268F, and of acetylcholine was unaltered on the gut treated with botulinum toxin, as shown in Figs. 9 and 10; in contrast, the previously equiactive doses of MBF not only failed to evoke a contraction but actually inhibited pendulum movements. In three of these experiments the "reversed" or inhibitory action of MBF was abolished by hexamethonium (Fig. 9 I).

Interaction with Atropine.—The observation that the motor response to nicotine of the rabbit's intestine is resistant to small doses of atropine which abolish the action of acetylcholine has been made by numerous workers in the past (for detailed references see Ellis and Rasmussen, 1951, p. 1). Furthermore it has been shown by Vogt (1943), von Euler (1945), and Ellis (1951) that the responses to other substances, likewise believed to be ganglion-cell-stimulating agents (NaCl, Na lactate, and piperidine), are also unaffected by atropine in rabbits. As first suggested by Vogt (1943), this phenomenon, which has been observed so far only in the rabbit's intestine but not in that of guinea-pigs or young kittens (Ambache and Edwards, 1951), appears to bear some resemblance to the atropine-resistance of certain parasympathetic cholinergic nerve-effects.

Ellis (1951) has suggested that the species differences in response to atropine referred to above may be of some use in the classification of drug actions. According to him, additional information as to whether an action is "muscarinic, nicotinic, or musculotropic" may be obtained by duplicate-testing on the atropinized intestines from two species, one of which must be known to exhibit atropine-resistance to nicotine, as in the rabbit, and the other not (guinea-pig or cat). Substances with a muscarine-like action would be antagonized by atropine 10^{-6} (Ellis, 1951) on both preparations; substances with a "musculotropic" action would not be antagonized by the same dose of atropine on either preparation; lastly, nicotine-like substances would be atropine-sensitive in intestinal preparations from guinea-pigs (or cats), but would exhibit some measure of atropine-resistance in rabbit gut. Our present experience with nicotine and MBF and the earlier experiments of Ambache and Edwards (1951) show that atropine-resistance in rabbits and guinea-pigs is itself subject to variation. Nevertheless, if allowance is made for this,

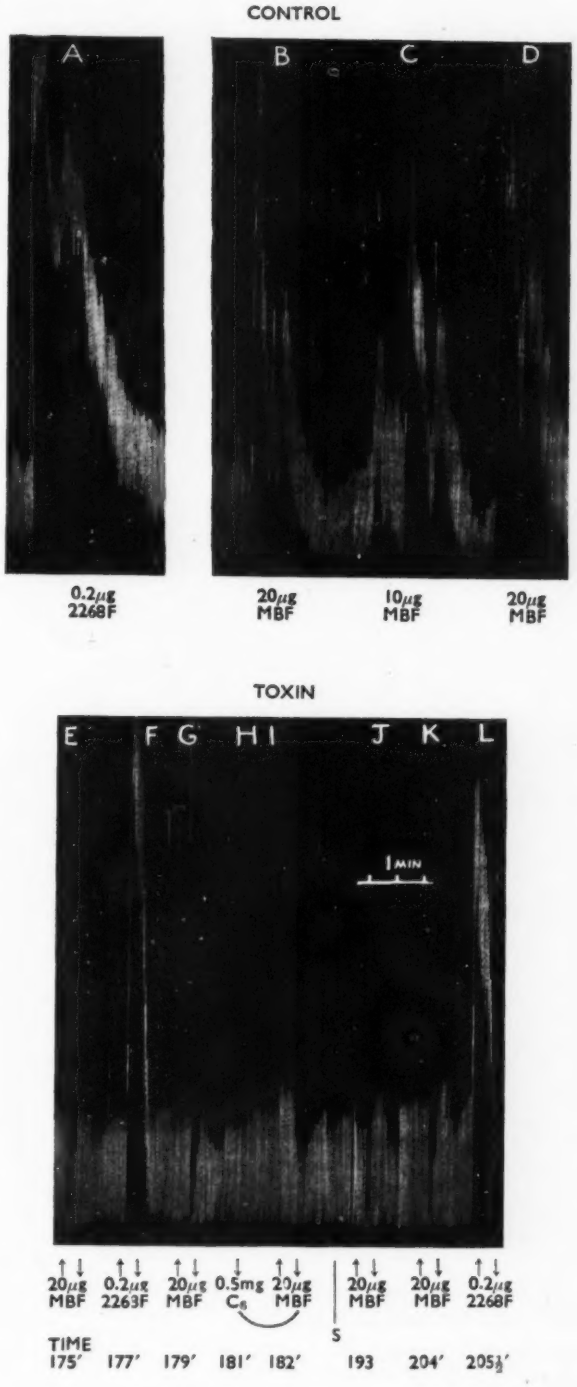


FIG. 9.—Reversal of the response to MBF by botulinum toxin Rabbit, 2.6 kg. *Top record* from a control segment of ileum (85 cm. from pylorus) excised before the injection of toxin. *Bottom record* from a segment 5–10 cm. lower, which was injected with 3 mg. of toxin A_2. The rabbit was killed 190 min. later. Time on the bottom record is in minutes from the moment of death. Drum stopped at S for 8 min. All drugs administered for 30 sec., except H. At A, F, and L, 0.2 μg. 2268F. At B, D, E, G, I, J, and K, 20 μg. of MBF. At C, 10 μg. of MBF. At H, 0.5 mg. of hexamethonium 60 sec. before I.

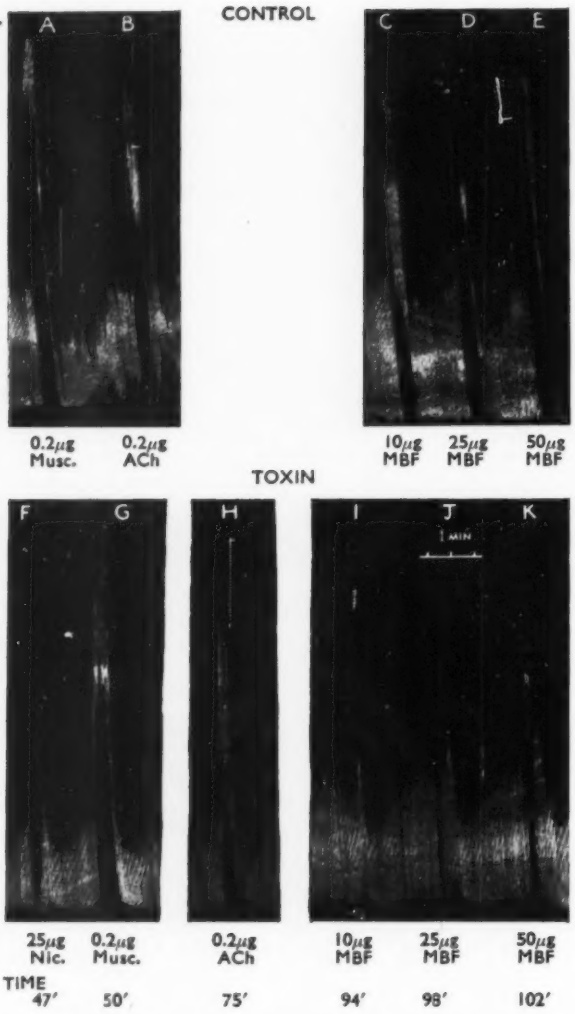


FIG. 10.—Specificity of the MBF-reversal by botulinum toxin. Rabbit, 3.4 kg. (coeliac and superior mesenteric ganglia excised 14 months previously). *Top record* from a control segment of ileum excised before the injection of toxin. *Bottom record* from an adjoining segment which was injected with 2.4 mg. of toxin A_2. Rabbit killed 85–90 min. after injection. Time in the bottom record in minutes from the moment of death. At A and G: 0.2 μg. muscarine. At B and H: 0.2 μg. acetylcholine. MBF at C and I, 10 μg.; at D and J, 25 μg.; at E and K, 50 μg. At F, 25 μg. nicotine.

a certain amount of information can be gleaned from duplicate tests of this kind. The results may be summarized by saying that the nicotine response was not invariably atropine-resistant in rabbits, but that, when it was so (in three of five preparations), the MBF response followed suit and persisted (though slightly reduced) in the presence of atropine 10^{-7} or 10^{-6}. But in guinea-pigs atropine 0.5–2×10^{-7} was sufficient to extinguish nicotine and MBF responses.

Because of the variations mentioned the atropine-resistance of MBF was studied in parallel with that of nicotine, and the two appeared to vary to the same extent. In detail the results on rabbits were as follows. In preparation No. 1 the contraction produced by 10 μg. of MBF persisted in the presence of atropine 10^{-6}, though reduced by 20%; an equivalent dose of muscarine was ineffective. In preparation No. 2, 20 μg. of MBF was matched by 20 μg. of nicotine and 0.4 μg. of muscarine (see Fig. 11). In the presence of atropine 10^{-7} the response to muscarine was abolished; that to nicotine and MBF persisted, though both were reduced to the same extent. In experiment No. 3 a match was obtained between 10 μg. of MBF and 8 μg. of nicotine; both effects were equally reduced, to 50% by atropine 10^{-7} and to 40% by atropine 10^{-6}. In two other experiments both the MBF and the nicotine effects were greatly reduced or abolished by atropine 0.5–2×10^{-7}.

In three of the four experiments on guinea-pig's ileum 0.5–1×10^{-7} of atropine completely abolished the response to equipotent doses of MBF and nicotine; this effect was reversible. As in the rabbit gut, the MBF response was blocked by hexamethonium. In the two experiments on kitten's ileum the actions of 2–20 μg. of MBF and of 2.5–10 μg. of nicotine were abolished by 0.5–2×10^{-7} of atropine.

DISCUSSION

It is clear that the mono- and dibromo- derivatives of choline phenyl ether possess intense nicotine-like actions not only on ganglia but also on skeletal muscle and on nicotine-sensitive axons in the skin. The motor action of these compounds on the longitudinal layer of the gut also resembles that of nicotine in the following respects: (1) An increase in dose may produce a decrease in effect, perhaps because of the onset of nicotine-like "paralysis"; (2) the effect is antagonized by hexamethonium; (3) it can be reversed by botulinum toxin; (4) the effect of these compounds is usually atropine-resistant in intestinal preparations from rabbits, but not from guinea-pigs or cats. All these properties would seem to point to an "indirect" action of these compounds on the gut through its motor nerve-supply, but there is no clue at present whether this action is entirely confined to the ganglion-cells or not. The fact that it is antagonized by hexamethonium is no longer decisive, since hexamethonium blocks nicotinic effects not only on ganglia but also at nerve-endings (Douglas and Gray, 1953). Hexamethonium can therefore no longer be used with absolute certainty to decide whether a nicotinic effect is due to stimulation at the ganglion-cell level or at nerve-endings. An action at both levels is theoretically possible with

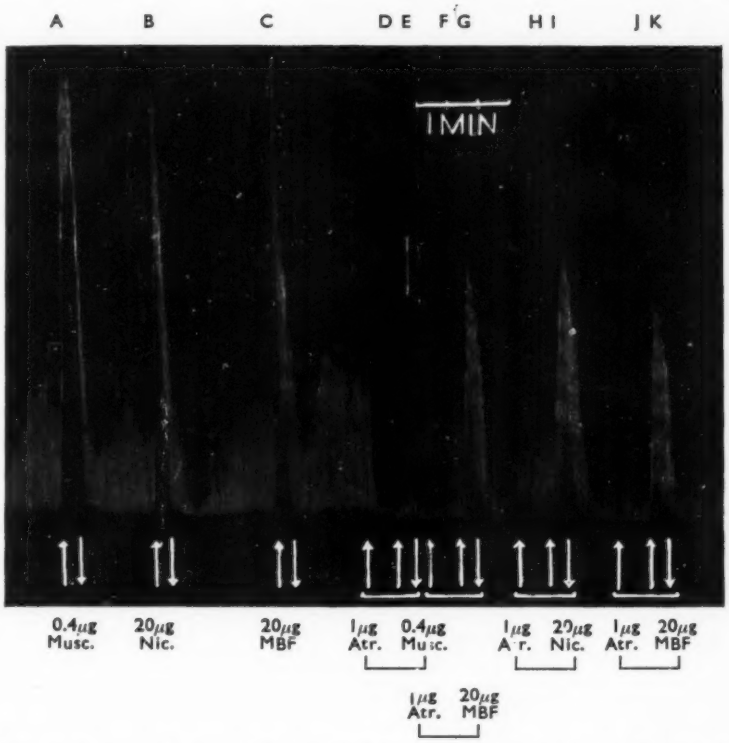


FIG. 11.—Example of atropine-resistance of the MBF response. Rabbit ileum 150 cm. below pylorus. Approximate matches between A, 0.4 μg. of muscarine; B, 20 μg. of nicotine; and C, 20 μg. of MBF. Atropine 1 μg. at D, F, H, and J for 60 sec. before repeating 0.4 μg. of muscarine at E, 20 μg. of nicotine at I, and 20 μg. of MBF at G and K. The action of muscarine is abolished by atropine, but the response to MBF and to nicotine persists, though reduced (both to a similar extent).

MBF and DBF (as with nicotine), since these drugs are capable, apart from stimulating ganglia, of exciting axon reflexes in certain ganglion-cell-free nerve-nets, for instance in the skin. There is still no evidence whether such an "axonic" action of nicotine and its congeners can or does occur at other nerve-endings in smooth muscle, but it is a possibility which cannot be overlooked.

SUMMARY

A number of nicotine-like actions of 3-bromo- and 3:5-dibromo-phenyl ethers of choline (MBF and DBF) are described, notably:

1. Their ability to excite skeletal muscle, as exemplified by the response of the frog's rectus abdominis (antagonized by *d*-tubocurarine) and by constriction of the pigeon's iris.

2. Stimulation and block of autonomic neurones either at the cell-body or at the nerve-endings. The action on ganglia has been examined on the perfused superior cervical ganglion of cats. That on nerve-endings (here termed the "axonic" action) can be conveniently studied in the human skin on the pilomotor endings. Both actions are blocked by hexamethonium.

3. The intestinal motor action, which can be interpreted in terms of nicotine-like stimulation of the motor neurones in the gut. This effect is blocked by hexamethonium and reversed by botulinum toxin. Its interaction with atropine varies in different species in the same way as that of nicotine. There is evidence of block with MBF in larger doses, as shown by the depression of nicotine-responses.

We wish to thank Dr. P. Hey for the gift of these compounds, and Miss Daphne Taylor for her technical assistance.

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THE ACTION OF NICOTINE AND ACETYLCHOLINE ON THE VESSELS OF THE RABBIT'S EAR

BY

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The action of nicotine on blood vessels is usually explained as being due to stimulation of sympathetic ganglia, as was shown by Langley (1901). However, Handovsky and Pick (1913) found that nicotine caused vasoconstriction when perfused through the vessels of the frog hindlegs (Läwen-Trendelenburg preparation), from which it appeared that nicotine might exert a more peripheral effect. They used a 1% solution, but in 1937 Loewi made similar observations using a 0.01% solution, and in 1946 Haimovici and Pick obtained vasoconstriction by injecting 5 μg. nicotine ; they observed that the effect was blocked by thiamine and thiazole. Later Haimovici (1948) showed that continuous perfusion with nicotine 1 in 50,000 caused initial constriction, but the rate of flow gradually increased and the injection of large doses of nicotine was then without effect. Haimovici found that the action of nicotine was unaffected by removal of the sympathetic chains and of the spinal nerves and that it persisted during perfusion with tetraethylammonium or with an extract of curare. He concluded that the action was peripheral to the postganglionic fibres and perhaps directly on the blood vessels.

The dilator action of small amounts of acetylcholine such as 5 μg. on the perfused vessels of the isolated rabbit ear was described by Dale in 1914, but in 1918 Reid Hunt stated that large amounts caused vasoconstriction both in the rabbit ear and in the vessels of the muscles of cats and of dogs ; in the rabbit ear he injected as much as 5 mg.; he concluded that acetylcholine had a constrictor action on blood vessels which was exerted somewhere " beyond the ganglia cells." In discussing these results Feldberg and Minz (1932) state that Bartosch and Nagel found that the constrictor action of acetylcholine was abolished by nicotine, though Hunt had found nicotine ineffective in this way. Further, Hirose (1932) observed that, when acetylcholine was injected into the femoral artery of a cat, the outflow of blood from the leg was diminished.

Feldberg and Minz made intra-arterial injections of acetylcholine into the splanchnic area of cats ; they prepared the cats by removing the adrenals, tying the abdominal aorta below the origin of the inferior mesenteric artery, and tying the inferior vena cava below the liver. They found that injection of acetylcholine into the aorta above the origin of the coeliac artery, atropine having first been given, caused a rise of blood pressure by splanchnic vasoconstriction. This effect was diminished but not abolished by removal of the solar plexus. They found the effect was abolished by large doses of nicotine, and thought it possible that acetylcholine exerted its constrictor action by stimulating ganglia placed more peripherally than the solar plexus and inferior mesenteric ganglia.

Burn and Robinson (1951) studied the effect of acetylcholine in the rabbit ear perfused with Locke's solution, and observed that in the first few hours of perfusion it dilated the vessels. They noted that, as the perfusion was continued, the dilatation caused by a given dose of acetylcholine declined and disappeared, and that at this stage a larger dose of acetylcholine caused a constriction. They found that, once a constrictor response to acetylcholine had set in, there was no reversal to a dilator action with any dose. They supposed that both the dilator and the constrictor effects were due to a direct action on the vessels. Burn and Dutta (1948) had previously observed that the constrictor effect of acetylcholine was reversed to a dilator effect when Priscol (2-benzyl-2-imidazoline hydrochloride) was added to the perfusion fluid.

The present work was begun in order to examine the effect of nicotine on the blood vessels, and in particular to observe whether it was modified by hexamethonium.

METHOD

Rabbit ears removed from the freshly killed animal were perfused with oxygenated Locke's solution according to the method described by Burn and Robin-

son (1951), using Stephenson's recorder (1948) to measure the outflow. When the ears were to be tested on the second day, the perfusion was continued overnight without interruption. In some experiments the skin was removed with a sharp scalpel, beginning with a flap on the convex side of the ear near the proximal end. Although it was often found that it was possible to remove the skin completely on the convex surface only, as much of the skin on the rest of the ear was removed as was possible, without damage to the rest of the ear.

All substances for injection were made as concentrated acidified solutions, which were of the following strengths; acetylcholine bromide 4 mg./ml., nicotine hydrogen tartrate 2 mg./ml., atropine sulphate 1 mg./ml., hexamethonium bromide 4 mg./ml. The final dilution of each substance was made in Locke's solution, just before each injection, and the *p*H was tested with Universal indicator. The doses of the injections are expressed in terms of the salts. When the response to any substance was a dilatation, a control injection of the same volume of Locke's solution was given to eliminate any dilator effect due to the volume of the injected fluid. All injections were contained in a volume of 0.05 ml. Locke's solution.

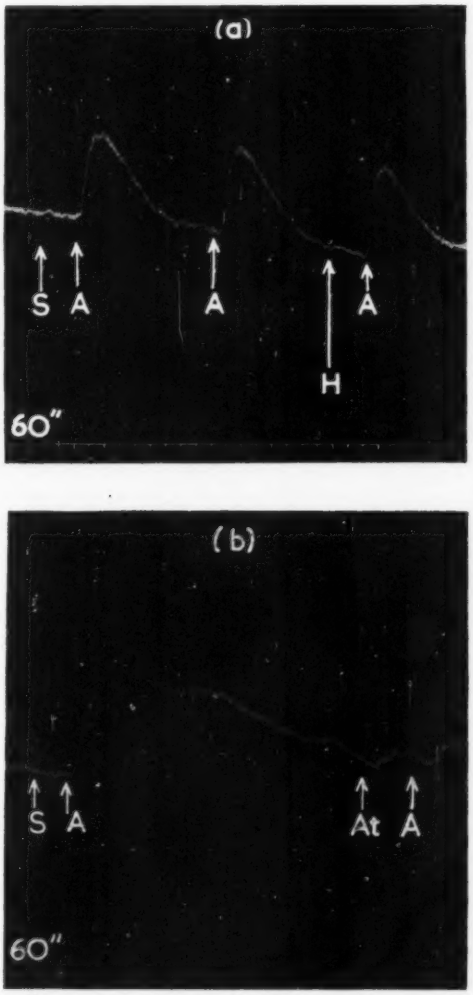

FIG 1.—(*a*) Outflow record from perfused rabbit ear; rise of the record indicates increase of outflow. S=Injection of 0.05 ml. Locke's solution. A=Injection of 2 μg. ACh in 0.05 ml. Locke's solution. H=Injection of 0.1 mg. hexamethonium. (*b*) S as in (*a*). A=Injection of 5 μg. ACh. At=Injection of 4 μg. atropine sulphate

RESULTS

Dilatation Caused by Acetylcholine.—As found by earlier workers the response to acetylcholine was initially a dilatation. This dilatation was not affected by hexamethonium, but was abolished by atropine (Fig. 1), which indicated that the dilator action of acetylcholine was muscarine-like. This observation was made in 8 trials in 4 preparations.

Constriction Caused by Acetylcholine and Nicotine.—The effect of a larger dose of acetylcholine on the ear after the perfusion had continued for some hours was different, for it caused a constriction which was blocked by hexamethonium as shown in Fig. 2*a*. The constrictor action of

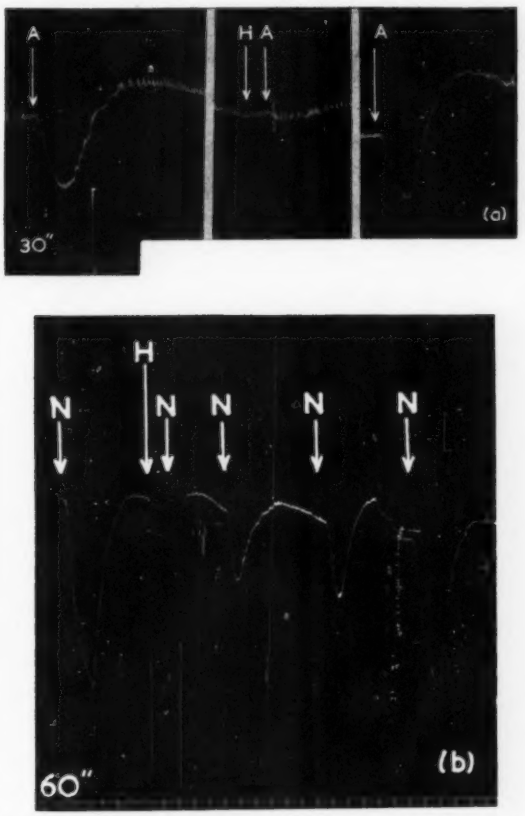

FIG. 2.—Record in this and subsequent figures as in Fig. 1. (*a*) At A, 0.1 mg. ACh was injected; at H, 0.1 mg. hexamethonium was injected. (*b*) At N, 25 μg. nicotine acid tartrate was injected; at H, 0.1 mg. hexamethonium was injected.

acetylcholine reappeared when the hexamethonium was washed out. This was observed in 7 trials in 4 preparations. Nicotine also caused a constriction (Fig. 2*b*), and this constriction was also blocked by hexamethonium. The constrictor action gradually returned as the hexamethonium was removed. The block of nicotine action was complete in 11 out of 14 trials and partial in 3 out of 14 trials ; 9 preparations were used. Nicotine, unlike acetylcholine, usually caused a constriction in the early stages of perfusion, but the constriction increased as the perfusion was continued.

These results suggested that the constrictor action of acetylcholine was a nicotine-like action.

Conversion of a Dilator Action into a Constriction.—If acetylcholine possessed both muscarine-like and nicotine-like actions in this preparation, the injection of atropine should convert the dilator action into a constrictor action under suitable conditions. This is shown in Fig. 3, in which a

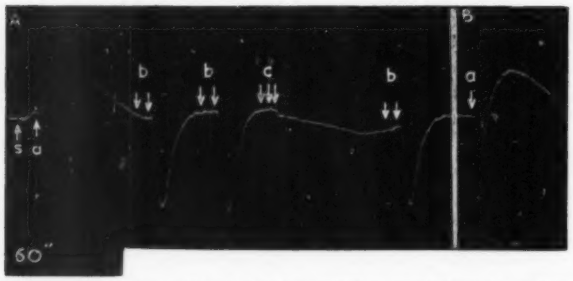

FIG. 3.—In A, at s, 0.05 ml. Locke's solution was injected; at " a," 20 μg. ACh was injected; at " b " 8 μg. atropine followed by 20 μg. ACh was injected; at " c," 0.1 mg. hexamethonium followed by 8 μg. atropine and then by 20 μg. ACh was injected. In B, at " a," 20 μg. ACh was injected.

moderately high dose of acetylcholine (20 μg.) caused a dilatation (Fig. 3a). When this dose of acetylcholine was repeated after injecting 8 μg. atropine it now caused a constriction (Fig. 3b). After the injection of 0.1 mg. hexamethonium and 8 μg. atropine, acetylcholine had no effect (Fig. 3c). The constrictor action of acetylcholine after atropine returned when the hexamethonium was washed out. In part B of Fig. 3 the effect of the same dose of acetylcholine alone is shown which was observed 1½ hr. later. At this point acetylcholine caused an initial constriction followed by a dilatation. The double response showed both nicotine-like and muscarine-like effects, the latter outlasting the former.

Mixed Effects of Acetylcholine and Nicotine.—When acetylcholine caused both constriction and dilatation, hexamethonium b'ocked only the constrictor action, and had no effect on the dilatation. Nicotine sometimes caused a dilatation after an initial constriction. As with acetylcholine, only the constriction was abolished by hexamethonium, while the dilatation was unaffected (Fig. 4).

The action of tetraethylammonium (TEA) on the constriction caused by nicotine and acetylcholine was similar to that of hexamethonium, though TEA often had an initial constrictor action of its own.

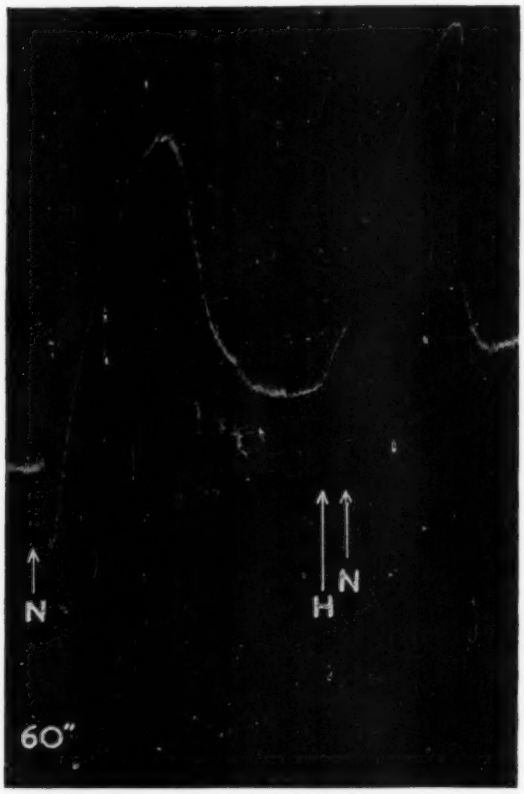

FIG. 4.—This preparation was unusual, because when it was fresh nicotine caused dilatation in 10 μg. and 20 μg. The above record, obtained on the second day, shows the effect of injecting 20 μg. nicotine at N; when 0.1 mg. hexamethonium was given first at H, the constrictor phase of the nicotine action was abolished.

Effect of a Reversing Agent.—Since Burn and Dutta (1948) had shown that the constrictor action of acetylcholine was reversed by Priscol, it seemed possible that acetylcholine and nicotine caused constriction by releasing adrenaline or a similar substance. The constriction caused by nicotine was also found to be reversed by Priscol (Fig. 5*a*). If the dilatation which nicotine caused in the presence of Priscol was due to the release of an adrenaline-like substance, then hexamethonium should abolish this response. Fig. 5*b* illustrates the finding that hexamethonium blocked the dila-

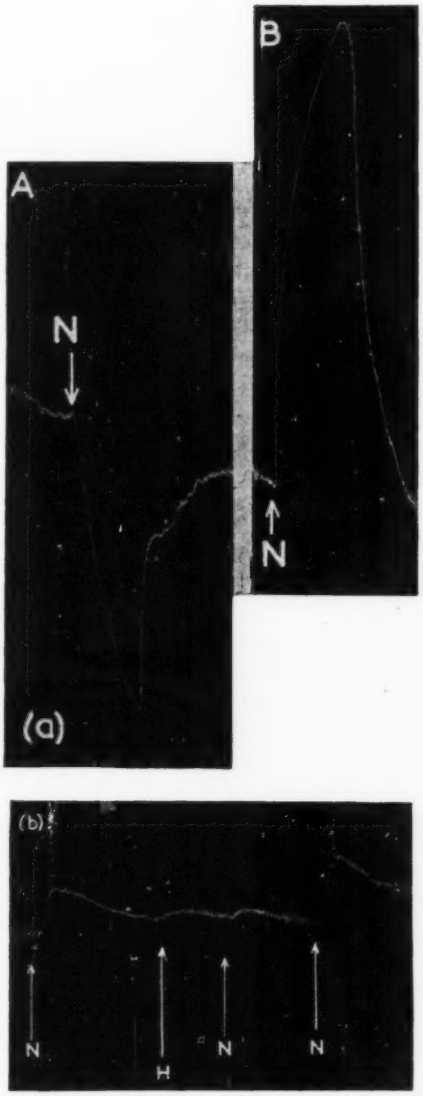

FIG. 5.—(*a*) In A the constrictor effect of injecting 5 μg. nicotine is shown. In B the ear was perfused with Locke's solution containing Priscol (20 mg. per l.). The injection of 1 μg. nicotine caused dilatation. (*b*) During perfusion of the ear with Priscol (10 mg. per l.) 10 μg. nicotine was injected at N. At H, 0.1 mg. hexamethonium was injected.

tation which nicotine caused in the presence of Priscol, and that as the hexamethonium was washed out the dilator action of nicotine returned.

Removal of the Skin.—From the results presented it appeared very likely that the site of the constrictor action of nicotine and acetylcholine was different from that of the dilator action of these two substances. I therefore decided to test the reaction of the vessels to nicotine and acetylcholine before and after the removal of most of the skin in the hope of separating these two sites anatomically. By this method it was possible to separate the dilator and constrictor actions of these two substances. Whereas in the ear with the skin intact the constriction caused by a given dose of nicotine or acetylcholine increased with the duration of the perfusion, after removal of the skin the constrictor action of both these substances was greatly reduced or absent and often a dilator response was seen. Records obtained before and after removal of the skin are shown in Fig. 6 (*a* and *b*). A dose of nicotine which caused constriction in the ear with the skin intact caused a dilatation in the same ear after removal of the skin. This dilatation which nicotine caused in the skinned ear, like the one which sometimes follows the constriction in the unskinned ear (Fig. 4), was not blocked by hexamethonium, which indicated that this was due to a direct action of nicotine. A similar result was obtained with acetylcholine (Fig. 6*b*). In one experiment the vessels of the skinned

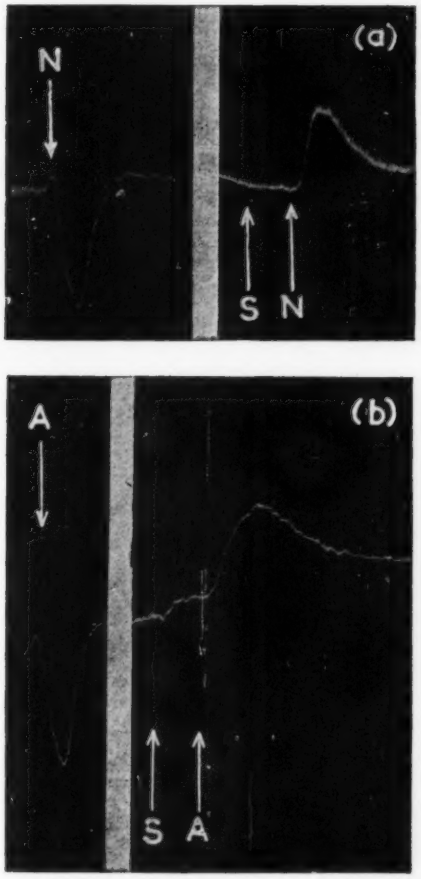

FIG. 6.—(*a*) On left is shown the constrictor action of 40 μg. nicotine. The skin was removed, and the next morning, as shown on the right, the injection of 40 μg. nicotine caused dilatation. S is control injection of perfusion fluid. (*b*) On the left is shown the constrictor action of 5 μg. ACh, at A. The skin was then removed. The next morning, as shown on the right, 5 μg. ACh caused dilatation.

ear responded with a dilatation to a dose of 0.0025 μg. (in 0.05 ml.) of acetylcholine, while the injection of the same volume of Locke's solution had no appreciable effect.

Discussion

The experimental evidence shows that both acetylcholine and nicotine can affect the vessels of the rabbit ear in two ways. Acetylcholine has a direct vasodilator action which is abolished by atropine ; it is seen in almost all perfused rabbit ears when the perfusion begins. In a small proportion of preparations nicotine also has a direct vasodilator action, though this is not abolished by atropine. These dilator effects are not affected by hexamethonium. When the perfusion is continued for some hours, the vasodilator effect of acetylcholine disappears or is masked because a constrictor effect appears. This constrictor action of acetylcholine is blocked by hexamethonium. With nicotine there is a similar change from a vasodilator to a vasoconstrictor effect in the few preparations in which the former is seen, and in the majority the initial vasoconstrictor effect increases as the perfusion continues. This vasoconstrictor action is blocked by hexamethonium.

The increase in the constrictor action of nicotine as the perfusion proceeds calls to mind a similar change which was observed in the rabbit auricles when isolated and beating in Locke's solution. When freshly prepared, nicotine had no effect on the rhythm except in large amounts ; when the auricles were tested 24 hr. later, after being kept overnight in Locke's solution at 4° C., they were sensitive to smaller amounts (Kottegoda, 1953).

There is a close parallelism between the action of acetylcholine on the isolated rabbit auricles and on the perfused rabbit ear vessels. In both preparations there are two effects. There is an inhibitory or vasodilator action which is abolished by atropine, and there is a stimulant or constrictor action which is blocked by hexamethonium. The stimulant action on the auricles is probably due to a release of an adrenaline-like substance, since Hoffman, Hoffman, Middleton, and Talesnik (1945) have shown that acetylcholine releases an adrenaline-like substance from the isolated and atropinized mammalian heart. There is reason to suppose that the constrictor action on the rabbit ear is also due to the release of an adrenaline-like substance, since this action is reversed to a dilator action by the presence of Priscol in the perfusion fluid.

The parallelism is not quite so complete for nicotine, since its inhibitory action on the auricles is believed to be mediated through cholinergic ganglion cells and its rarely observed dilator action on the ear vessels is not abolished by atropine. However, the stimulant action of nicotine on the auricles appears to be similar to its constrictor action on the vessels.

When the ear has been perfused until all traces of dilator response to acetylcholine is lost and constriction is regularly obtained, the removal of the skin lays bare a network of vessels covering the cartilage which react to acetylcholine and also to nicotine by vasodilatation. After removing the skin it is usually necessary to leave the ear for some hours before this response is obtained. The sensitivity to acetylcholine then is sometimes extreme, vasodilatation being observed with so little as 2.5 mμg. acetylcholine.

The removal of the skin may remove the vessels which are responsible for the constrictor response, leaving behind those which are responsible for the initial dilator response which may have been put out of action by steadily increasing oedema, or the removal of the skin may remove ganglion cells or chromaffin tissue on which acetylcholine or nicotine may act to release an adrenaline-like substance, which is responsible for the constriction. It is difficult to choose between such alternatives. Experiments are now in progress to determine the effect of nerve degeneration on this action, and when the results are available the position should be clearer.

It is interesting to note in conclusion that as long ago as 1890 Langley and Dickinson found that nicotine caused constriction followed by dilatation in the rabbit ear when observations were made in the whole animal. They also found that after large doses of nicotine subsequent doses caused only dilatation.

Summary

1. Nicotine causes vasoconstriction in the perfused vessels of the isolated rabbit ear. This constrictor action is blocked by hexamethonium and tetraethylammonium.

2. When Priscol is added to the perfusion fluid, nicotine causes dilatation. This dilatation is blocked by hexamethonium.

3. It seems probable that both these effects of nicotine are due to the release of an adrenaline-like substance.

4. Acetylcholine causes dilatation in the freshly perfused ear, but when the perfusion is continued on the second day it causes constriction. The

dilator effect is blocked by atropine, but not by hexamethonium, while the constrictor action is blocked by hexamethonium (and tetraethylammonium) but not by atropine. It is already known that the constrictor action of acetylcholine is reversed to a dilator action by Priscol.

5. It seems probable that the constrictor action of acetylcholine, like that of nicotine, is due to the release of an adrenaline-like substance.

6. When a preparation is taken in which both nicotine and acetylcholine exert a constrictor action, the removal of the skin leaves a network of vessels on the cartilage in which both substances cause dilatation.

7. There is a close similarity in the two actions which acetylcholine exerts in the rabbit ear vessels and the two actions it has on the isolated rabbit auricles.

This work was done during a period of study leave from the Faculty of Medicine, University of Ceylon.

I am deeply indebted to Professor J. H. Burn for his guidance and encouragement throughout this investigation.

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THE ACTION OF DYES, ANTIBIOTICS, AND SOME MISCELLANEOUS COMPOUNDS AGAINST *PLASMODIUM BERGHEI*

BY

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(RECEIVED OCTOBER 30, 1952)

After it had been shown that *Plasmodium berghei* was sensitive to a number of antimalarial drugs (Thurston, 1950) its sensitivity to some dyes, antibiotics, and miscellaneous compounds was tested by a similar technique. Mice were infected with a smaller inoculum than the one used in the previous experiments, because it was found that light infections of *P. berghei* were more susceptible to the action of drugs than heavy infections. It has previously been reported that advanced infections of *P. berghei* were more difficult to cure than earlier ones (Baldi and Rocca, 1951), and that light infections of *P. gallinaceum* were more susceptible to the action of drugs than heavy infections, whether the inoculum consisted of parasitized erythrocytes (Davey, 1946) or of sporozoites (Greenberg, Trembley, and Coatney, 1950).

METHODS

Mice, ranging in weight from 15–20 g., were infected with an inoculum containing 1,000,000 parasites in 0.2 ml. citrated blood, injected intraperitoneally. Drugs were usually given intraperitoneally once daily for four days, commencing five to six hours after inoculation. Thin blood films, stained with 5% Giemsa, were examined on the fifth day and on the seventh day.

Untreated controls had an average infection of 1–5% of the erythrocytes parasitized on the fifth day. The minimum effective dose of a drug was taken to be the smallest dose which on the fifth day reduced the mean infection level to one-fiftieth of that of the controls. A compound was said to show slight activity if, when the maximum tolerated dose was given, the parasitaemia was significantly less than that of the controls but yet was not reduced to one-fiftieth of that figure.

The inoculum and resulting infection rate were lower than those used in the preliminary tests of antimalarial drugs against *P. berghei* (Thurston, 1950), because it was found that the same dose of drug showed much greater activity against a low infection than against a high one. For example, 0.2 mg. of mepacrine per 20 g. mouse cleared the blood for two days when the controls had a mean infection level of 2%, but the same dose only reduced the mean parasitaemia to 28% when the controls had a mean infection of 38%. A drug which was only slightly active at the maximum tolerated dose might easily be missed if tested against too severe an infection.

RESULTS

The results of chemotherapeutic tests with some dyes, antibiotics, and miscellaneous compounds are given in Table I. In this table the figures for the activity against *P. gallinaceum* have been taken from Wiselogle (1946) unless other references are given. It should be noted that drugs were administered by mouth to chicks infected with *P. gallinaceum*, whereas they were given intraperitoneally to mice infected with *P. berghei*. The two sets of figures are therefore not completely comparable although they give a good indication of the degree of activity against each infection. The figures for *P. gallinaceum* are quoted from blood inoculation tests and not from sporozoite infection tests. The quinine equivalent is the ratio

$$\frac{\text{Minimum effective dose of quinine}}{\text{Minimum effective dose of drug under assay}}$$

DISCUSSION

Dyes.—Methylene blue was at one time used in the treatment of human malaria although it had only slight specific action on the parasites (Werner, 1914). It was found that the introduction of a basic dialkylamino group into the side chain resulted in greatly increased activity (Schulemann, 1932). Pamaquin and mepacrine were synthesized during further experiments with similar basic side chains.

Mudrow-Reichenow (1951) showed that methylene blue was active against *P. berghei*. During the present experiments it proved to be the most

TABLE I

THE ACTIVITY OF DYES, ANTIBIOTICS, AND MISCELLANEOUS COMPOUNDS TESTED AGAINST *P. BERGHEI*, COMPARED WITH THEIR ACTIVITY AGAINST *P. GALLINACEUM*

Compound	Maximum Tolerated Dose mg./ 20 g. Mouse, i.p.×4	Activity Against *P. berghei* Minimum Effective Dose mg./ 20 g. i.p.×4	Activity Against *P. berghei* Quinine Equivalent	Activity Against *P. gallinaceum* Quinine Equivalent	Chemical Name
Quinine	2·0	1·0	1·0	Minimum effective dose 3·0 mg./100 g. b i d.×4 oral	
Proguanil	0·2 (b.i.d.)	0·2 (b.i.d.)	5·0	8·0 (Davey, 1946)	
Sulphadiazine ..	5·0	0·005	500	0·8	
Dyes:					
Methylene blue	1·0	0·3	3·3	0·06 oral	3 : 7-*bis*(Dimethylamino)phenazathionium chloride
Toluidine blue ..	1·0	0·5	2·0	0·04 diet	3-Amino-7-dimethylamino-2-methyl phenazathionium chloride
Thionin blue ..	2·0	1·0	1·0		3-Dimethylamino-7-ethylmethylamino phenazathionium chloride
Methylene violet (Bernthsen)	2·0	1·0	1·0	0·15 ,,	7-Dimethylamino-3-phenthiazone
Azure A ..		1·0	1·0	0·04 ,,	3-Amino-7-dimethylaminophenazathionium chloride
Methylene green	1·0	Slight activity	<1·0	<0·06 ,,	3 : 7-*bis*(Dimethylamino)-6-nitro-phenazathionium chloride
Nile blue ..	1·0	1·0	1·0	<0·03 oral	5-Amino-9-diethylaminobenzo(a)phenazoxonium hemisulphate
Meldola's blue..	0·3	Slight activity	—	<0·04 diet	9-Dimethylaminobenzo(a)phenazoxonium chloride
Brilliant cresyl blue	5·0	2·0	0·5	<0·03 oral (*P. lophurae*)	7-Amino-3-diethylamino-2-methyl phenazoxonium chloride
Gallamine blue	20	Inactive	—	<0·02 diet	1-Carbamyl-7-dimethylamino-3 : 4-dihydroxy phenazoxonium chloride
Thionin ..	5·0	Slight activity	<0·2	0·04 diet	3 : 7-Diaminophenazathionium chloride
Neutral red ..	2·0	Inactive	—	<0·3 oral	3-Amino-7-dimethylamino-2-methylphenazine chloride
Methylene violet (R.R.A.)	0·3	Slight activity	—	2·0 diet	3 : 7*bis*(Dimethylamino).5-phenyl phenazine chloride
Rhodamine B ..	2·0	,, ,,	<0·5	<0·03 oral (*P. lophurae*)	9(*o*-Carboxy phenyl)-3 : 6-*bis* (diethylamino)-xanthylium chloride
Gentian violet ..	0·2	Inactive	—	<0·4 diet	Hexamethyl *p*-rosaniline hydrochloride
Antibiotics:					
Penicillin ..	>10,000 units i.p. >100,000 units oral	Inactive	—	Inactive	
Chloromycetin..	>10	Inactive	—	Inactive at 10 mg./ 100 g (this experiment)	
Streptomycin ..	>10	,,	—	Inactive (*P. lophurae*)	
Aureomycin ..	2·0	0·5	2·0	0·25 (Coatney *et al.*, 1949)	
Terramycin ..	3·0	1·0	1·0	Inactive at 10 mg./ 100 g (this experiment)	
Miscellaneous compounds:					
Neoarsphenamine	1·0	Slight activity	—	0·03	
Tartar emetic ..	0·5	0·5	2·0	<0·4 oral	
Anthiomaline ..	3·0	1·5	0·67	<0·02 ,,	
Fuadin ..	10	1·5	0·67	Inactive	
Marfanil ..	>30	Slight activity	<0·03	<0·02 oral	
D.2. Polyoxyethylene ether	25	Inactive			
p-Aminosalicylic acid	4·0	,,	—		
p-Chloroaniline	20	,,	—		
p-Bromoaniline	3·0	,,	—		
p-Aminobenzoic acid	>20	,,	—		
Folic acid ..	>10	,,	—		
Methionine ..	>10	,,	—		
Nicotinamide ..	>10	,,	—		

All figures for *P. gallinaceum* have been taken from Wiselogle (1946) unless other references are given.

active of the series of related dyes. In the phenazathionium compounds it would appear that substitution in the amino groups, i.e., increased potential basicity, was accompanied by an increase in activity. In methylene blue both the amino groups carry two methyl radicles while thionin, in which both the amino groups are unsubstituted, had the lowest antimalarial activity. Methylene green resembles methylene blue, but contains a nitro-group in the 6– position ; it had only slight activity against *P. berghei.* Toluidine blue, thionin blue, methylene violet (Bernthsen), and azure A have various radicles on one or both of the amino groups.

The phenazathionium compounds and phenazoxonium compounds were considerably more active against *P. berghei* than against *P. gallinaceum.* The order of activity was not the same, for methylene violet (Bernthsen) was less active than methylene blue against *P. berghei,* whereas it was 10–40 times as active as any other compound against *P. gallinaceum.*

The other dyes which were tested against *P. berghei* had different types of structure ; they were either inactive or had very slight activity. When tested against *P. gallinaceum* they were more active than the dyes of the methylene blue and nile blue series.

Antibiotics.—Levaditi and Vaisman (1950) found that aureomycin was slightly active against *P. berghei* and that penicillin (100,000 units per mouse) and chloromycetin (40 mg. per mouse) were moderately active when the drugs were given by mouth once daily for three days. Streptomycin was inactive. Penicillin was inactive in human malaria, although there was some indication that it potentiated the activity of quinine (Deshmukh, 1947). Aureomycin was slightly active against *P. vivax,* but did not give radical cures (Cooper, Coatney, Imboden, and Jeffery, 1949).

In the present experiments, penicillin, chloromycetin, and streptomycin were inactive against *P. berghei.* Both aureomycin and terramycin were active against *P. berghei,* aureomycin being twice as active as terramycin. Aureomycin was more active against *P. berghei* than against *P. gallinaceum.* Terramycin was inactive against *P. gallinaceum.*

Miscellaneous Compounds.—Arsenicals were at one time used in the treatment of malaria, though with little effect. Neoarsphenamine was inactive against *P. berghei* when given by mouth (Mudrow-Reichenow, 1951). It was active when given subcutaneously, and the activity could then be antagonized by dimercaprol (Black, 1951). In the present experiments, neoarsphenamine showed only very slight activity when given intraperitoneally.

Tartar emetic, anthiomaline, and fuadin (compounds which contain antimony) were more active against *P. berghei* than against *P. gallinaceum.*

Although *P. berghei* is very sensitive to the action of sulphadiazine it was only slightly affected by marfanil. This drug differs from the sulphonamides, which are active antimalarials, in having the amino group separated from the benzene ring by a methylene group, and unlike them its antibacterial activity is not antagonized by *p*-aminobenzoic acid.

The polyoxyethylene ether (D.2), which is active against *Mycobacterium tuberculosis* (Cornforth, Hart, Rees, and Stock, 1951), and *p*-aminosalicylic acid, which also possesses considerable antibacterial activity *in vivo,* were both inactive against *P. berghei.*

p-Chloroaniline and *p*-bromoaniline were possible degradation products of proguanil and its bromo-analogue ; both were inactive.

p-Aminobenzoic acid, folic acid, methionine, and nicotinamide were inactive against *P. berghei.* Galliard and Lapierre (1950) reported that folic acid had slight antimalarial action against *P. berghei.* Marshall, Litchfield, and White (1942) found slight activity in *p*-aminobenzoic acid when tested against *P. gallinaceum.*

These experiments again emphasize the differences between species of malaria parasites in their sensitivity to drug action and the value of testing potential antimalarials against as many species as possible.

Summary

1. Tests for chemotherapeutic activity were performed against light infections of *Plasmodium berghei,* as these were more sensitive than heavy infections.

2. Methylene blue was the most active of a series of related compounds. Aureomycin and terramycin were active against *P. berghei* ; penicillin, chloromycetin, and streptomycin were inactive. Antimonials were more active against *P. berghei* than against *P. gallinaceum* ; neoarsphenamine had very little effect against *P. berghei.* Marfanil was very slightly active against *P. berghei.*

p-Aminosalicylic acid, D.2, *p*-chloroaniline, *p*-bromoaniline, *p*-aminobenzoic acid, folic acid, methionine, and nicotinamide were all inactive.

I wish to thank Dr. F. Hawking for encouragement in this work and Miss D. Ryder and Miss M. Cameron for technical assistance.

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THE PROPERTIES OF *n*-PROPYL 3 : 5-DI-IODO-4-PYRIDONE-N-ACETATE (PROPYLIODONE)

BY

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The suggestion to use organic iodine compounds for visualizing the bronchial anatomy was first made by Waters, Bayne-Jones, and Rowntree (1917). They reported experiments on dogs in which a mixture of iodoform and olive oil had shown considerable promise. Human bronchography first became practicable in 1922 when Sicard and Forestier employed iodized poppy-seed oil. Chemically similar contrast media used in bronchography have been iodized sesame or arachis oils and ethyl di-iodobrassidate. Viscous aqueous solutions of diodone were first used by Titus, Tafel, McCullen, and Messer (1938, 1939). Contrast media of this type were designed to overcome the delayed lung clearance, characteristic of iodized oils, by using the concentrated aqueous solution of an organic salt. Their use involves administration of a very hypertonic solution which causes instantaneous "flooding" and a fleeting bronchogram.

It occurred to the authors that a suspension of a contrast medium which was slightly soluble in body fluids should overcome the major disadvantages of both iodized oils and diodone solutions, viz., delayed lung clearance and hypertonicity respectively.

We have examined several contrast media for their solubility in saline and their toxic effects on the lung. Practically all were rejected for one or both reasons. Diodone was the least irritant, but its solubility was too great. Since diodone is the diethanolamine salt of 3 : 5-di-iodo-4-pyridone-N-acetic acid, a search was made for a suitable derivative of the present acid. Among the derivatives examined were the alkyl esters, which appeared to satisfy both initial requirements. Finally the *n*-propyl ester was chosen, and the following is an account of the pharmacological and metabolic investigations carried out with it.

MATERIALS AND METHODS

The normal propyl ester of 3 : 5-di-iodo-4-pyridone-N-acetic acid,

(propyliodone, Dionosil brand), is a white crystalline solid, m.p. 186–7° C. (with decomp.). It is readily sterilized by dry heat and will withstand a temperature of 150° C. for at least two hours without undergoing any detectable changes in composition. It is slightly soluble in saline and serum. The solubilities in water at 15, 35, and 95° C. are 0.014, 0.020, and 0.110 g./100 ml. respectively.

The pulverized crystals can be suspended either in aqueous media with a mixture of sodium carboxymethyl-cellulose and a suitable wetting agent, or in vegetable oils. The terms "aqueous propyliodone" and "oily propyliodone" employed throughout the text refer to 50% w/v suspensions in an aqueous medium and in arachis oil respectively.

Toxicity

Intratracheal.—The aqueous and oily suspensions were injected into the trachea (0.4 ml. per kg. body weight) at 15-minute intervals into rabbits anaesthetized with a combination of sodium pentobarbitone and chloralose. The animals tolerated four to five doses before becoming asphyxiated.

Two groups each of 18 rabbits were injected through the crico-thyroid membrane, the first receiving aqueous propyliodone and the second control group the aqueous vehicle. A third group of 18 rabbits was injected with oily propyliodone. Two more groups of 12 rabbits each were injected, one with iodized poppy-seed oil and the other with a viscous preparation of diodone (50% w/v). Each rabbit received 1 ml. of the appropriate substance. At intervals of 16 hours, 3 days, 5 days, 2 weeks, and monthly up to 5 months after the injection, two rabbits from each of the first three groups were killed, and the lungs were examined both macro-

scopically and histologically. Sections of the lungs from all 36 rabbits in the first two groups were fixed and stained for sodium carboxymethyl-cellulose by a modified Feyrter method (Vischer, 1951). The lungs from the rabbits in the third group were fixed, and frozen sections were prepared and stained with "Oil Red 4B." Macroscopic examinations were carried out at intervals on animals from the fourth and fifth groups.

Bronchography in Rabbits.—Comparative bronchographic studies were carried out in rabbits with the new compound, iodized poppy-seed oil, and a viscous preparation of diodone (50% w/v). Both aqueous and oily propyliodone were investigated lest the aqueous suspension should provoke excessive coughing.

The appropriate contrast medium (0.6 ml.) was administered to rabbits anaesthetized with sodium pentobarbitone. The medium was injected either into the trachea through the crico-thyroid membrane or directly into the bronchial tree by means of an oral catheter. Local anaesthesia was not practised, as it would depress the cough reflex, which serves as a guide to the cough-promoting properties of the various preparations. Not unexpectedly it was found that the aqueous suspension was somewhat more irritant than the oily one, although the extra coughing induced was not excessive and in no way impaired the quality of the bronchogram. Iodized oils caused as much coughing as the oily suspension. Diodone invariably produced violent and persistent coughing. The progress in the lung of the various media was followed on a fluoroscopic screen.

Animal Metabolism Studies

Excretion Rate and Serum Levels in the Rabbit.—Aqueous propyliodone (0.8 ml.) was administered to female rabbits by the intratracheal or oral route. To facilitate excretion studies the compound was labelled with I_{131}. Hourly blood samples were taken for the first six hours from the animals placed in metabolism cages, and the urines were collected until they were no longer radioactive. At the end of the experiment the animals were killed and the faeces, alimentary tract, and major organs were examined for radioactivity.

Chromatographic Identification of Metabolite.—Urine samples were collected from rats that had previously received oral doses of labelled propyliodone. The undiluted urines were spotted on filter paper (Whatman No. 2) and developed with saturated sodium bicarbonate by the ascending technique. After drying, the paper strips were examined in ultra-violet light. The same strips were also used to develop radio-autographs.

Human Metabolism Studies

Excretion Rate after Bronchography.—Aqueous propyliodone (22 ml. of 50% w/v suspension) was injected into a patient through the crico-thyroid membrane. During the next 82 hours urine was collected. The volumes were recorded and aliquots analysed for total iodine by the method of White and Rolf (1940). Sputum samples were not collected. All urine samples were tested for free iodine (starch) and iodide ion (starch-nitrite).

Isolation and Identification of Metabolite in Human Urine.—A second patient was injected by the crico-thyroid technique with 20 ml. of oily propyliodone. It was not possible to collect his urine for more than 28 hours. The excreted metabolite was identified as 3:5-di-iodo-4-pyridone-N-acetate ion as follows (Baker and Briggs, 1943):

250 ml. of urine (3.73 mg. I/ml.) were made alkaline (*p*H 10) and filtered. The filtrate was saturated with sodium chloride and the resulting precipitate separated on a centrifuge. The mother liquors contained 8 μg. I/ml. The precipitate was washed twice with saturated brine and dissolved in distilled water. After adjusting the resultant solution to *p*H 3.5 with HCl, white crystals separated. Final purification of the material was achieved by dissolving the crystals in boiling water and cooling the solution in a refrigerator overnight. The resultant solid was separated, dried to constant weight, and analysed. The equivalent weight of the acid was determined by titrating an aliquot against standard NaOH solution. The m.p. was compared with that of the laboratory standard. Isotope-dilution experiments were carried out on a bulked sample of the urine.

Excretion Rate after Oral Administration.—Solid propyliodone (10 g.) was ingested by a volunteer and urine samples were collected during the next 10 hours. The samples were assayed for organic iodine. Here again isotope-dilution experiments were performed on a bulked sample.

RESULTS

Toxicity

Intratracheal.—After administration of aqueous propyliodone and the aqueous vehicle, macroscopic signs of lung irritation were apparent at 16 hours and reached a maximum at three days. After two weeks the lungs appeared normal. They showed the same degree of congestion after the propyliodone or its aqueous vehicle, but in no instance was the congestion greater than was observed in the animals dosed with iodized oil. Microscopic examination revealed a few scattered foci of round cell infiltration in the interstitial tissue adjoining the small bronchi of the rabbits killed 16 hours, three days, five days, and one month after instillation of propyliodone. Small deposits of carboxymethyl-cellulose could be demonstrated during the first few days in the mucosa and cartilage of small bronchi and in the alveoli. Traces were seen in the few macrophages of the inflammatory foci in the interstitial tissue. In no animal could sodium carboxymethyl-cellulose be demonstrated after five days.

Macroscopic examination of the lungs after administration of oily propyliodone showed that congestion was greatest at three days and absent after two weeks. In none of the rabbits was the degree of congestion greater than was seen in animals treated with iodized oil. Droplets of non-opaque oil were present in the bronchi and alveoli up to five days after injection, but later there was no evidence of residual oil.

In those rabbits which received iodized poppy-seed oil marked congestion of the bronchial mucosa had developed after 16 hours and was most apparent in the small bronchioli. After one week the degree of congestion was undiminished and the lungs still contained appreciable amounts of iodized oil visible to the naked eye. Presumably the lung changes continued for varying periods depending on the persistence of the oil. Compared with iodized oils, vegetable oils are cleared rapidly from the healthy rabbit lung; iodine is slowly liberated by the former. There may be some connexion between these two phenomena.

Diodone preparations caused immediate flooding of the lung tissue with serous fluid, and by the time this had been resorbed (2 hours) erythema and swelling of the mucosa were pronounced. Slight interstitial oedema persisted for five days.

Oral.—Propyliodone was administered as a single oral dose of 18 g./kg. to mice. No immediate toxic symptoms were observed and thereafter growth rates were normal. Fluoroscopic examination revealed a radio-opaque bladder and no contrast medium in the colon.

Intravenous.—Aqueous propyliodone was injected intravenously into forty mice in a dose of 120 mg./kg. The mice were disorientated at first, but recovered within an hour. Their subsequent behaviour was normal. The LD50 for mice by this route was 300 mg./kg. Death was almost certainly due to occlusion of blood vessels.

Subcutaneous, Intramuscular, and Intraperitoneal.—Injection of mice and rabbits by these routes with large doses of aqueous or oily propyliodone (1 ml.) produced no signs of systemic toxicity. Invariably unabsorbed contrast medium was found at the injection site. Absorption by these routes must be extremely slow.

Bronchography in Rabbits

Figs. 1, 2, and 3 are of bronchograms taken immediately, two hours, and three days respectively after administration of propyliodone. It will be seen that no alveolar filling had occurred after two hours and that all contrast in the lungs had disappeared within three days. The bronchograms shown are typical of both the aqueous and oily media. Although many experiments have been performed in rabbits with these two preparations, alveolar filling has not yet been encountered. On many animals a further bronchographic examination was made after three days (i.e., when all traces of the first dose had disappeared), and invariably the second bronchogram was identical with the first. Presumably serial bronchography should present no difficulties in the human patient.

Figs. 4, 5, and 6 were obtained with iodized poppy-seed oil. They show all the characteristics of the immediate, fifteen-minute, and three-day bronchograms respectively. When coughing occurred during administration, it was immediately succeeded by alveolar filling.

All efforts to obtain a satisfactory bronchogram with a viscous solution of diodone (50% w/v) were unsuccessful.

Animal Metabolism Studies

Excretion Rate and Serum Levels in the Rabbit.—Figs. 7 and 8 show the urinary excretion rates of I_{131} in the animals dosed by the intratracheal and oral routes respectively. In both a minimum of 95% was recovered within 72 hours. In no instance during the first six hours was the total iodine concentration in the serum greater than 0.01% (calculated as propyliodone). At the end of the experiment the faeces, alimentary tract, and major organs were not radioactive. Apart from traces in the lung and urinary tract, no radioactive iodine remained in the animals that had been injected by the intratracheal route.

Chromatographic Identification of Metabolite.—On examining the paper strip in ultra-violet light, a single fluorescent purple spot (Rf 0.68) was observed, corresponding with sodium 3:5-di-iodo-4-pyridone-N-acetate. Radio-autographs developed from these paper strips revealed the presence of a single radioactive spot coinciding with the fluorescent spot. When chromatographed under these conditions, propyliodone remained at the site of application.

Human Metabolism Studies

Excretion Rate after Bronchography.—It will be seen in Fig. 9 that a steady elimination of iodine-containing metabolite, totalling 50%, occurred in the first 72 hours. The absence of any lag in excretion was shown by the appearance of organic iodine in the first voiding, suggesting that no secondary accumulation of the compound or its metabolite occurs in the body. All tests for iodine or iodide ion in the urine gave negative results.

Rabbit's bronchograms after administration of propyliodone

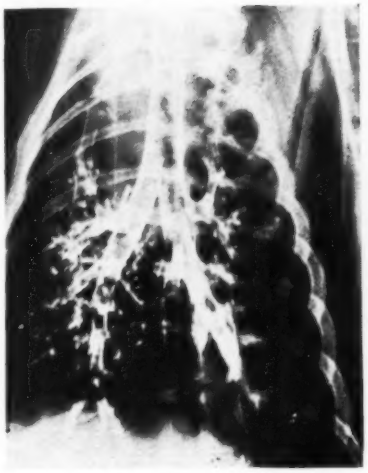

FIG. 1.—Immediately.

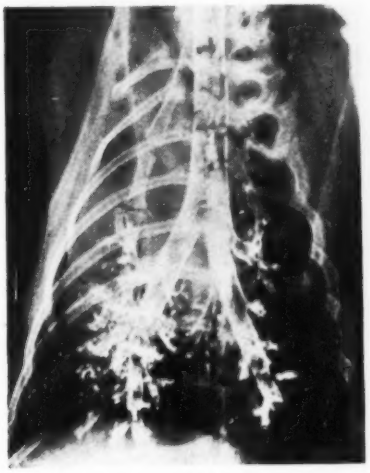

FIG. 2.—2 hours.

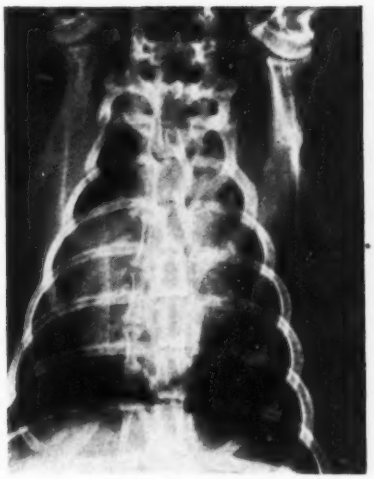

FIG. 3.—3 days.

Rabbit's bronchograms after administration of iodized poppy-seed oil

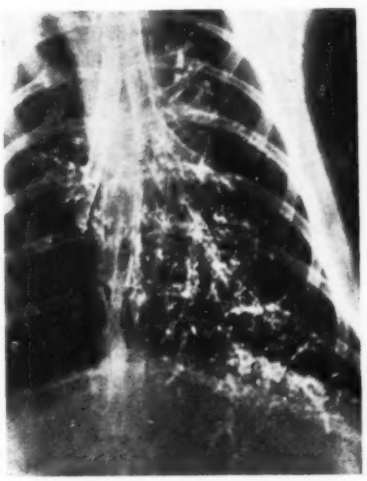

FIG. 4.—Immediately.

FIG. 5.—15 minutes.

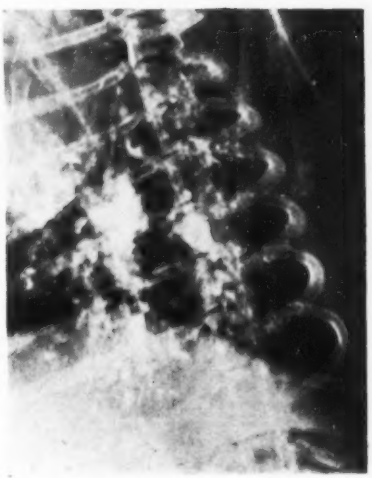

FIG. 6.—3 days.

[*Facing p. 168.*

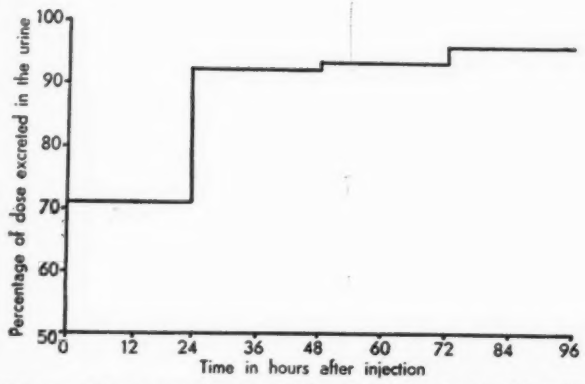

FIG. 7.—Urinary excretion in rabbits of 3:5-di-iodo-4-pyridone-N-acetate ion after intratracheal administration of propyliodone.

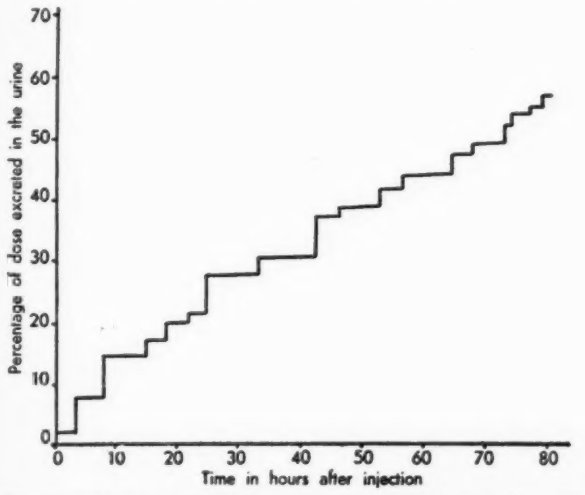

FIG. 9.—Urinary excretion in man of 3:5-di-iodo-4-pyridone-N-acetate ion after intratracheal administration of propyliodone.

Isolation and Identification of Metabolite in Human Urine.—The weight of pure acid finally obtained was 754 mg. (I=62.73%) which represented a recovery from the urine of 50%. Analysis gave C, 20.40; H, 1.06; N, 3.25; I, 62.73% (calculated for $C_7H_5O_3NI_2$: C, 20.76; H, 1.24; N, 3.46; I, 62.68%). The equivalent weight of the acid was 403 (calculated for $C_7H_5O_3NI_2$, 405). The crystals melted at 242° C. (decomp.), whilst the laboratory standard melted at 243° C. No depression of the mixed melting point was observed. The isotope dilution experiments confirmed the presence in the urine of only one metabolite containing iodine.

Excretion Rate after Oral Administration.—After the ingestion of 10 g. of propyliodone, no nausea or other untoward reactions were observed. The rate of organic iodine excretion by the kidney is shown in Fig. 10. Here again the isotope dilution experiments proved that only one iodine-containing metabolite (the anion of the parent acid) was present in the urine.

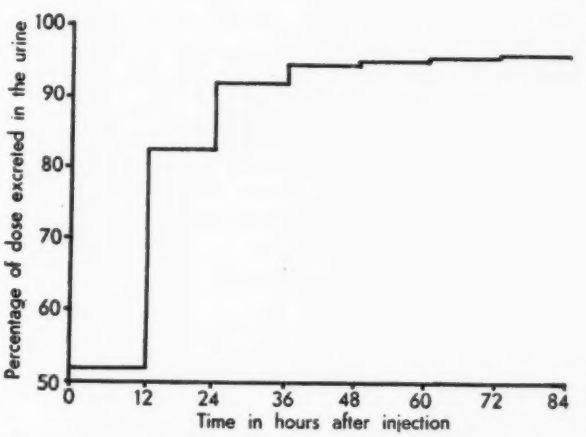

FIG. 8.—Urinary excretion in rabbits of 3:5-di-iodo-4-pyridone-N-acetate ion after oral administration of propyliodone.

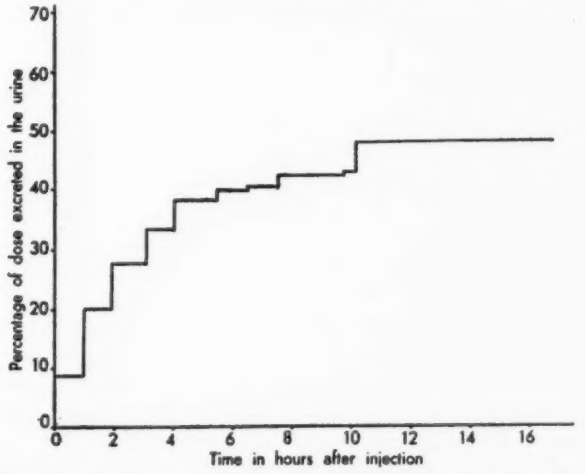

FIG. 10.—Urinary excretion in man of 3:5-di-iodo-4-pyridone-N-acetate ion after oral administration of propyliodone.

DISCUSSION

Unlike all other contrast media known at the time of its introduction, iodized poppy-seed oil was non-irritant in the lung and could be easily introduced into the trachea. It has found universal acceptance without serious rival for nearly thirty years. Nevertheless, it has serious disadvantages which detract from its value and restrict its use. Alveolar filling, commonly encountered, especially if coughing occurs during administration, results in obscured bronchograms. This would not be so serious if repeat bronchography were possible. Depending as it does on a combination of postural drainage, expectoration, and phagocytosis, complete lung clearance is often long and unpredictably delayed, making serial bronchograms difficult to obtain.

Water-soluble contrast media were introduced into bronchography in an attempt to ensure rapid lung clearance. The actual duration of such media in the bronchial tree is a matter of a few minutes. This does not permit an unhurried fluoroscopic examination, which is generally regarded as essential for successful bronchography. Since these solutions are grossly hypertonic, the lungs rapidly become flooded: coughing ensues, and the contrast medium is diluted with bronchial secretions.

There is, therefore, a need for a bronchographic agent without these disadvantages. It was argued that a suspension of a slightly soluble contrast medium should be completely eliminated from the lungs within a few days, and furthermore it should not cause osmotic disturbances.

It would appear from the experiments outlined in the text that propyliodone may fulfil both these requirements. Furthermore, it does not cause alveolar filling in the healthy rabbit lung, even after coughing. It remains to be seen whether the same lack of alveolar filling is observed in the diseased human lung. Our experience with aqueous and oily propyliodone in anaesthetized rabbits suggests that the aqueous suspension is more irritant (i.e., cough promoting) than iodized poppy-seed oil. Although oily propyliodone is a suspension, it does not appear to be more irritant than iodized oil. Presumably the oil acts as a lubricant for the crystals. Undoubtedly aqueous propyliodone will necessitate careful premedication if undue coughing is to be avoided in man.

The human excretion rate experiments suggest that the use of propyliodone should enable serial bronchographic examinations to be made at intervals of a week or so. Lung clearance rates will, without doubt, vary from one patient to another, but complete clearance should be obtained within a week or ten days.

The experimental evidence suggests that propyliodone is hydrolysed in the bronchial tree and the resulting organic anion is then rapidly removed by the kidney. Since neither iodine nor iodide ion could be detected in the urine after intratracheal or oral administration of propyliodone, it follows that it should be possible to administer the material to iodine-sensitive patients. Iodized oils should not be administered to such patients (Robertson and Morle, 1951; Lichter, 1951; Theodos, 1952). Because of the breakdown in the lung, it has been suggested that iodized oils be precluded in cases complicated by tuberculosis (Amberson and Riggins, 1933). This argument should not apply to propyliodone.

Summary

1. Aqueous and oily suspensions of normal propyl 3:5-di-iodo-4-pyridone-N-acetate (propyliodone) have been compared in rabbits with iodized oil and diodone. Propyliodone did not enter the alveoli, and clearance was complete within a few days.

2. No toxic symptoms were observed after intratracheal or oral administration of propyliodone. Elimination after subcutaneous, intraperitoneal, and intramuscular injection was extremely slow.

3. The congestion produced in rabbit lung after injection of aqueous or oily propyliodone was similar in degree to that observed with iodized oil, but of a shorter duration. Sodium carboxymethyl-cellulose could not be demonstrated for longer than five days after injection. Similarly non-opaque oil was absent after intervals of more than five days.

4. Animal experiments with propyliodone labelled with I_{131} and human metabolic studies have shown that, after intratracheal or oral administration, the compound is completely hydrolysed and eliminated via the kidney. Iodine and iodide ion are absent in the urine.

We wish gratefully to acknowledge the help we have received from Sir Harold Graham Hodson, Dr. J. N. Pattinson, Dr. J. K. Ross, Dr. C. G. Whiteside, and Sister Stone, of the Middlesex Hospital, where the first clinical trials were undertaken, and to Dr. F. Pygott and Dr. M. G. Scott, of the Central Middlesex Hospital; without the collaboration of nurses and patients at both hospitals the human metabolic studies could not have been conducted. In particular we wish to thank Drs. Pattinson and Whiteside for their stimulating advice throughout our work. We also wish to thank Miss H. M. Sharpe, B.Sc., for valuable assistance in the laboratory.

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THE ANTAGONISM OF HISTAMINE AND THE ANAPHYLACTIC RESPONSE BY PHENYLPYRIDYLALLYLAMINES

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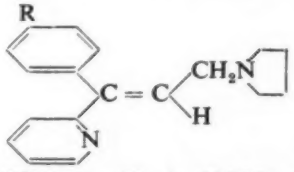
A moderate degree of antihistamine activity was found in a series of diphenylallylamines (White, Green, and Hudson, 1951) and in some dithienylallylamines (Green, 1953), and investigation of the related phenylpyridylallylamines made by Adamson and Billinghurst (1950) has shown that many of these latter compounds are powerful antihistamines. They can exist in two steroisomeric forms. The antihistamine activity is confined mainly to the *trans* isomer, as in *trans*-l-(4′-chlorophenyl)-l-(2′-pyridyl)-3-pyrrolidinoprop-l-ene maleate (Adamson, Barrett, Billinghurst, Green, and Jones, 1951). This compound, known by the code number 405C49, has been shown to have a powerful antihistamine action in man (Bain, 1951).

A description of the pharmacological properties of 405C49 and the most active member of this series, *trans*-l-(4′-methylphenyl)-l-(2′-pyridyl)-3-pyrrolidinoprop-l-ene hydrochloride (295C51) (Adamson, Barrett, Billinghurst, and Jones, 1953), in laboratory animals is presented in this paper.



In 405C49, R = Cl ; in 295C51, R = CH_3

RESULTS

Effects on Histamine Action

Guinea-pig Ileum.—The compounds were compared for relaxation of histamine-induced spasm of isolated guinea-pig ileum by the method described previously (Green, 1953). The activities, relative to that of 405C49, were calculated from the percentage relaxation of histamine spasm within three minutes. In Table I are shown the mean values obtained by comparing the compounds on the ileum of each of several guinea-pigs. 295C51 is twice as active as 405C49 ; the latter is equal to mepyramine and twice as active as chlorprophenpyridamine (Chlortrimeton).

TABLE I

RELATIVE ANTIHISTAMINE ACTIVITIES COMPARED WITH THAT OF 405C49 IN ISOLATED GUINEA-PIG ILEUM

Compound	No. of Comparisons	Activity	
		Mean	Limits of Error p=0·95
405C49	—	1·0	—
295C51	6	2·1	1·8–2·4
Mepyramine ..	4	1·1	0·97–1·3
Chlorprophenpyridamine ..	3	0·54	0·42–0·69

Histamine Asthma.—The compounds were compared, using 405C49 as standard, by observing the incidence of "asthma" in groups of guinea-pigs exposed to a histamine aerosol for five minutes, thirty minutes after injecting the antihistamines intraperitoneally. A total of 220 animals received 405C49, and it was estimated that the dose protecting 50% of pigs was 0.094 mg./kg., with limits for p=0.95 of 0.07–0.12 mg./kg. Compound 295C51 was at least five times as active as 405C49, and the mean estimate of the activity ratio for these two compounds was 8.6 (Table II). Mepyramine and chlorprophenpyridamine may be

TABLE II

RELATIVE ANTIHISTAMINE POTENCIES BY THE HISTAMINE ASTHMA TEST IN GUINEA-PIGS AT 30 MINUTES AFTER INTRAPERITONEAL INJECTION AND ONE HOUR AFTER ORAL ADMINISTRATION

Limits of error for p=0·95 in brackets

Compound	Intraperitoneal		Oral	
	No. of Pigs per Compound	Activity	No. of Pigs per Compound	Activity
405C49 ..	220	(1·0)	80	(1·0)
295C51 ..	90	8·6 (5·0–15·0)	80	2·9 (1·5–5·8)
Mepyramine	170	1·5 (1·0–2·1)	80	1·6 (0·79–3·1)
Chlorprophenpyridamine ..	20	1·8 (0·56–6·1)	80	3·7 (1·8–7·1)

slightly more active than 405C49, but the differences were not significant. No significant differences were detected in the duration of action with doses of the four compounds which showed equal activity at thirty minutes after injection, but there was a tendency for the duration to be shorter with 405C49 than with the other compounds.

All four compounds are less potent when given orally than when given intraperitoneally. For example, the dose of 405C49 preventing asthma in 50% of guinea-pigs exposed to a histamine aerosol one hour after oral administration was 2.3 mg./kg. (limits, p=0.95, 1.4–3.8), i.e., about ten times the intraperitoneal dose. The mean estimates of the relative potencies of the four antihistamines examined (Table II) were not the same for oral administration as for intraperitoneal injection, but the only significant difference was between the 295C51 : 405C49 ratios (p=0.01–0.02). When given by mouth the activities of 295C51 and chlorprophenpyridamine were significantly greater than that of 405C49, and probably greater than that of mepyramine.

Histamine Fall of Blood Pressure.—295C51 and 405C49, like other antihistamines, reduce but do not abolish the depressor action of histamine in the anaesthetized dog. Significant reductions in the histamine effect have been observed with doses as low as 0.01 mg./kg. of 295C49 or 0.05 mg./kg. of 405C49. Our experiments have not been sufficient in number to determine the relative activity of these two compounds, but they indicate that 405C49 may be about as active as mepyramine and that 295C51 may be considerably more active. Very large doses (5 to 10 mg./kg.) not only greatly reduce the histamine response but lower the blood pressure and reduce its response to acetylcholine.

Gastric Secretion.—Wood (1948) observed that mepyramine increased the rate of gastric secretion of cats during a slow infusion of histamine. Using a similar procedure, but taking a continuous record of secretion, we found that 295C51 had the same type of action. A single intravenous dose of 1 mg./kg. was without effect, but after 5 mg./kg. intravenously the rate of secretion doubled in three minutes and reached a maximum in 30 minutes, when its volume was 230% and its total acidity 330% of the pre-injection level. The increased rate of secretion was maintained for over an hour.

Anaphylaxis in the Isolated Ileum

Guinea-pigs were sensitized by intrahepatic injection, at the same time but at different sites, of two volumes of 0.05 ml. of a 10% solution of egg albumen. This procedure, which Barrett (1950) used with horse serum, gave a high incidence of anaphylaxis in guinea-pigs, or strips of smooth muscle from them, when tested with antigen 10 to 16 days later. The action of two or more concentrations of the antihistamines were compared on anaphylactic contracture of strips of isolated guinea-pig ileum (suspended in aerated Tyrode Ringer at 37–39° C.) on the same day using several strips from the same pigs. They were added to the organ bath three minutes before the antigen (egg albumen, 4×10^{-5}). Each anaphylactic contraction was expressed as a percentage of maximal by comparing its extent with that due to a large concentration of histamine (10^{-6}). In the absence of antihistamine the contraction due to the antigen, although taking longer to develop, was usually about maximal; only when this was so was the ileum used for comparing the effect of antihistamines.

The antihistamines greatly retarded the rate of anaphylactic contraction at concentrations much less than those which limited its final extent (Fig. 1). The inhibitory action was proportional to the dose, but usually only very high concentrations (10^{-5} or more) eliminated the response to antigen In Fig. 2 are shown the number of guinea-pigs (in groups of eight) the ileum of which gave a response to antigen of 50% or less of maximal, at each of five concentrations of 405C49, 295C51, and mepyramine. These compounds were approximately equiactive. Very much larger amounts of each were required to limit the response to antigen of the ileum from some animals than in that from others, the range for 50% inhibition lying between 10^{-5} and 3×10^{-7}. These amounts are very much greater than those which are needed to eliminate the spasmogenic action of large concentrations of histamine applied externally to the ileum.

Anaphylaxis in Vivo.—405C49, 295C51, and mepyramine were injected intraperitoneally in doses of 0.4 to 50 mg./kg. into sensitized guinea-pigs, 30 minutes before injecting 0.1 ml. of a 10% solution of egg albumen into the saphenous vein under local anaesthesia (0.1 ml. of 5% procaine). The results are summarized in Fig. 3. When no antihistamine was given nearly all the guinea-pigs developed severe "asthma" within 2–3 minutes, and most of them died in 5–10 minutes. Autopsy revealed typical severe emphysema and in some animals vascular engorgement of the gastrointestinal tract. In contrast, few of the antihistamine-treated animals developed severe asthma or died within 10 minutes. Those animals which succumbed within this time showed a similar postmortem appearance to that of the control animals. Although the antihistamines protected the guinea-pigs to a very large extent against the early mani-

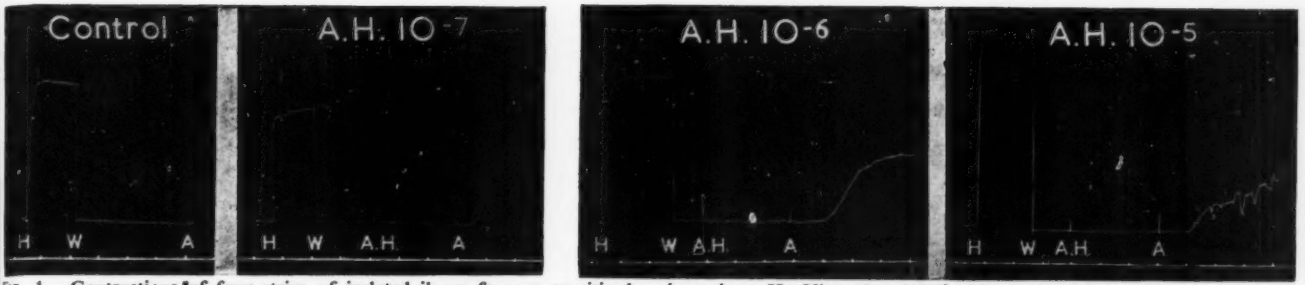


FIG. 1.—Contractions of four strips of isolated ileum from a sensitized guinea-pig. H=Histamine (10^{-6}), W=wash with the drum stopped for 10 minutes, A.H.=antihistamine 295C51 at various concentrations, A=antigen (egg albumen, 4×10^{-5}). Time scale in minutes.

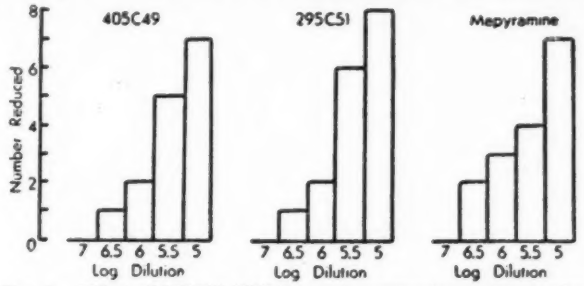


FIG. 2.—Effect of 405C49, 295C51, and mepyramine on the anaphylactic response of isolated guinea-pig ileum. The number of pigs out of eight whose contractures were reduced to 50% or less at various log. dilutions of the antihistamines.

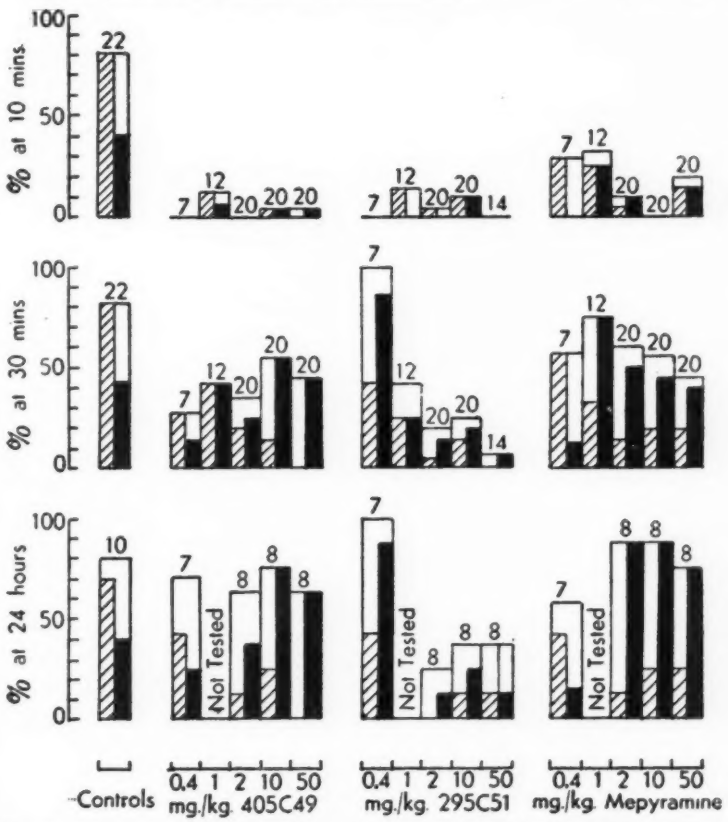


FIG. 3.—The percentage mortality in groups of sensitized guinea-pigs (number used shown above each column) at intervals after testing with antigen (i.v.), preceded by various doses of antihistamines (i.p. 30 minutes previously). The heights of the shaded and black portions show respectively the percentages dying with severe emphysema and with severe vascular congestion of the intestine.

festations of anaphylactic shock, they did not, with the exception of the higher doses of 295C51, prevent later collapse and/or dyspnoea in the majority of animals. At the end of 30 minutes nearly all the animals receiving the higher doses of 295C51 were still alive and mainly in good clinical condition, whereas about half the animals in the 405C49 and mepyramine groups had already died. Twenty-four hours later the mortality was substantially less than that of the controls only in the groups receiving 2 to 50 mg./kg. of 295C51 (during the latter part of this period the single doses of antihistamines given would be exerting little effect). Vascular congestion of the gastro-intestinal system and of other tissues was a more prominent feature than severe emphysema of the lung in the delayed deaths (Fig. 3). It was also found, by sacrificing a number of animals, that vascular engorgement of the intestines was marked in many survivors 30 minutes after administering antigen.

Other Properties

Toxicity.—In mice the LD50s (and limits of error for p=0.95) of 295C51, 405C49, and mepyramine were respectively 21 (18–25), 24 (20–27), and 41 (38–44) mg./kg. intravenously, and 300 (250–360), 180 (160–220), and 170 (160–190) mg./kg. subcutaneously. The LD50 of chlorprophenpyridamine subcutaneously was 260 mg./kg. (limits 210–320).

A dog was unaffected by 5 mg./kg. of 295C51 intravenously, but almost immediately after the injection of a further 10 mg./kg. 15 minutes later, severe tremors, hyperexcitability, and dyspnoea followed by hyperpnoea occurred. These effects disappeared within two hours. The same symptoms followed by severe clonic con-

vulsions were caused by 50 mg./kg. of 405C49 intraperitoneally ; the convulsions were completely controlled by pentobarbitone sodium, subsequent recovery from anaesthesia being uneventful. In dogs anaesthetized with pentobarbitone sodium the fatal intravenous doses of 405C49 and 295C51 have varied between 15 and 40 mg./kg. Such amounts caused precipitous falls in blood pressure, apnoea, hyperpnoea, and within 1–2 minutes complete respiratory arrest. There were no tremors or convulsions.

Daily subcutaneous injection of 10 mg./kg. of 405C49 or 295C51 in newly weaned rats, for a period of nine weeks, did not affect their growth, behaviour, erythrocyte counts or macroscopic appearance at autopsy. Dr. D. J. Trevan found no histological evidence of damage due to the drugs in the lungs, livers, spleens, and kidneys of these animals.

Blood Pressure and Breathing.—Large doses of 405C49 and 295C51 lower the blood pressure of cats and dogs under pentobarbitone sodium. In dogs the pressure falls about 10–30 mm. Hg with 2 to 4 mg./kg. and 60 mm. Hg with 10 mg./kg. of 405C49 ; it falls about 10 to 50 mm. Hg with 1 to 5 mg./kg. and 90 mm. Hg with 30 mg./kg. of 295C51 intravenously. The fall is of short duration with doses below 5 mg./kg., but more persistent with larger amounts. The administration of atropine, or severing both vagosympathetic trunks, did not affect the depressor action of 405C49 in the dog.

Given intravenously in doses of 1 to 10 mg./kg., 405C49 and 295C51 greatly increase the respiratory movements of dogs under pentobarbitone. With the lower doses the effect is small and temporary, but with higher doses, after brief apnoea, the respiratory volume is usually at least trebled for up to half an hour or more.

Action on the Bowel.—The compounds 295C51 and 405C49 had very little effect on the activity of the bowel. Strips of isolated rabbit ileum suspended in aerated Tyrode Ringer at 37–39° C. ceased to contract spontaneously and showed loss of tone when treated with 405C49 and 295C51 at the high concentration of 5×10^{-5}. Concentrations of 10^{-5} caused only slight inhibition, sometimes preceded by a transient increase in motility. 405C49, 20 mg./kg. subcutaneously, did not affect the propulsion of a charcoal meal in rats. Intravenous doses as high as 6 mg./kg. or more of 295C51 reduced the motility of the duodenum and ileum of a dog under pentobarbitone sodium.

Temperature.—The rectal temperatures of resting rabbits fell slightly in some but not all animals, but never more than 1° C., when given 10 mg./kg. of 405C49 or 295C51 intravenously.

Analgesia.—405C49 and 295C51, in doses of 50 mg./kg., had no significant effect on the pain threshold of rats when tested by the pressure method of Green and Young (1951).

Local Anaesthesia.—Both 405C49 and 295C51 caused corneal anaesthesia (to touch with a rounded glass rod) lasting 15 to 45 minutes when applied as 5% solutions, buffered at *p*H 6, to the rabbit eye for one minute. The duration of action was similar to that of mepyramine or chlorprophenpyridamine under the same conditions, when tested in groups of six rabbits.

Atropine-like Properties.—Like mepyramine and chlorprophenpyridamine, 405C49 and 295C51 were remarkably free from atropine-like effects. In mice none of these compounds dilated the pupil except when given in doses nearing the amounts causing fatal respiratory arrest. When tested on isolated guinea-pig ileum the amounts required to reduce spasm due to acetylcholine at a concentration of 5×10^{-7} were approximately a thousand times those which inhibit spasm due to the same concentration of histamine. 405C49, for example, gave 50% relaxation of spasm within three minutes at 7.2×10^{-6} (mean of two tests) when acetylcholine 5×10^{-7} was used, and at 5.6×10^{-9} (mean of 31 tests) against histamine spasm. The estimated activities of 405C49, 295C51, and chlorprophenpyridamine were respectively 0.0006, 0.0015, and 0.0006 times that of atropine sulphate. They reduced the response to acetylcholine in dogs under pentobarbitone sodium only in doses which themselves lowered the blood pressure, i.e., only at very high doses.

Discussion

The antihistamine action of the phenylpyridylallylamines, 405C49 and 295C51, are of the same order of magnitude and as specific as those of mepyramine and chlorprophenpyridamine ; the relative potencies of these four compounds in the guinea-pig vary with the method of measurement. 295C51 is considerably more active than the other compounds in relaxing histamine spasm of the guinea-pig ileum *in vitro*. The greater activity is more pronounced when given intraperitoneally in preventing the so-called histamine asthma in the guinea-pig ; but when given orally under similar conditions, while its activity is still great, it is now equalled by that of chlorprophenpyridamine

(Table II). In the present experiments intraperitoneal mepyramine is nearly as effective as chlorprophenpyridamine, in agreement with the results of Feinberg, Malkiel, Bernstein, and Hargis (1950). They also found that intraperitoneally the two compounds gave a similar degree of protection against intravenous histamine. In contrast with these findings and the present observation that, when given orally in the asthma test, mepyramine is about half as active as chlorprophenpyridamine, Margolin and Tislow (1950) report that mepyramine given orally has only 5.6% of the activity of chlorprophenpyridamine in protecting against intravenous histamine. The results in guinea-pigs therefore are by no means uniform. For this reason there can be only a broad parallelism between the activities of antihistamines in guinea-pigs and those in man. In reducing the response to intracutaneous histamine in man, 405C49 and chlorprophenpyridamine are about equally potent (405C49 being quicker and shorter acting) (Bain, 1951), while 295C51 is about five times as active (Bain, personal communication). Our results in guinea-pigs are similar. On the other hand, the finding that mepyramine has only 8% of the activity of 405C49 or chlorprophenpyridamine in man (Bain, 1951) contrasts with the results in guinea-pigs described here and by Feinberg, Malkiel, Bernstein, and Hargis (1950), but not with those of Margolin and Tislow (1950).

Dale (1948) distinguishes between the actions of intrinsic and extrinsic histamine. While antihistamines readily antagonize the effects of extrinsic histamine, the action of intrinsic histamine, such as is released during the anaphylaxis of smooth muscle, is more difficult to suppress (Loew, 1947). Many antihistamines have been examined, using several kinds of smooth muscle and different experimental conditions, and it has always been reported that the amount of antihistamine required to inhibit the anaphylactic response is far greater than that which blocks the action of added histamine. In our experiments on the anaphylactic response of isolated guinea-pig ileum to egg albumen it was found that, although the antihistamines (405C49, 295C51, and mepyramine) limited the final extent of anaphylactic contraction only in relatively high concentrations, the amount of antihistamine which caused marked delay in the onset and progress of spasm was comparable with that which blocked the effect of added histamine, and far less than that required to modify the effects of acetylcholine or barium. The height of the anaphylactic contraction was to some extent inversely related to the concentration of antihistamine in contact with the ileum. The concentration of antihistamine required for the same degree of inhibition varied greatly in the different pigs, in some animals 3×10^{-7} being sufficient to halve the contraction. It seems therefore that antihistamines have a very pronounced inhibitory action on the anaphylactic contraction of smooth muscle, and that this inhibition varies in degree with the concentration of antihistamine and the amount of histamine liberated by the antigen-antibody reaction. However, very large concentrations of antihistamine are required completely to eliminate the response to antigen, and these amounts are of the same order as those which inhibit the contraction caused by barium and acetylcholine. This might be due to the high concentration of intrinsic histamine released during the antigen-antibody reaction at the cell membranes. There are, however, a number of experimental findings (Schild, 1936, 1949; Kellaway and Trethewie, 1940; Schild, Hawkins, Mongar, and Herxheimer, 1951) which suggest that the anaphylactic contracture of smooth muscle is not entirely due to histamine release. Kellaway and Trethewie (1940) postulated that a "slow reacting substance," liberated by the antigen-antibody reaction, was partly responsible for the anaphylactic contraction of smooth muscle, and showed that this substance was released more slowly than histamine. This "slow reacting substance" may be responsible for causing the delayed slow contraction of sensitized smooth muscle treated with antigen in the presence of concentrations of antihistamine sufficient to inhibit the rapid histamine contraction.

In the intact animal the antihistamines 405C49, 295C51, and mepyramine are only of limited effectiveness in controlling anaphylactic shock, 295C51 being considerably more effective than the other compounds. All three compounds greatly delay the onset of symptoms and in adequate doses reduce the incidence and severity of asthma and emphysema of the lung, but no single dose has succeeded in eliminating these effects completely. The observations support the generally accepted view that early asthma and emphysema is due to bronchoconstriction caused by liberated histamine, but the later emphysema must be due either to insuperable amounts of intrinsic histamine or to some other factor. As previously reported by Williamson (1936), the main effect in guinea-pigs dying late in anaphylaxis is not emphysema of the lung but congestion and haemorrhage in the gastro-intestinal tract and other tissues. This latter

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effect was scarcely influenced by any dose (0.4 to 50 mg. subcutaneously) of mepyramine or 405C49, nor by any but perhaps the highest doses of 295C51. It must therefore be supposed that the vascular component of anaphylaxis is due either to very large amounts of intrinsic histamine (it is known that antihistamines are of limited effectiveness in reducing the fall of blood pressure caused by histamine) or to another factor. Incidentally, it is not possible to produce the severe vascular congestion of the intestine seen in anaphylactic pigs by very large doses of histamine (10 to 1,000 mg./kg. subcutaneously) in either the normal or antihistamine-treated guinea-pig.

Summary

1. *Trans*-l-(4′-chlorophenyl)-l-(2′-pyridyl)-3-pyrrolidinoprop-l-ene maleate (405C49) and *trans*-l-(4′-methylphenyl)-l-(2′-pyridyl)-3-pyrrolidinoprop-l-ene hydrochloride (295C51) show antihistamine actions of the same order of magnitude and as specific as those of mepyramine and chlorprophenpyridamine. The relative activities of these four compounds vary in different tests. In guinea-pigs, 295C51 is easily the most active in relaxing histamine spasm of the ileum *in vitro* and, when given intraperitoneally, in preventing histamine asthma ; given by mouth, its activity is equalled by that of chlorprophenpyridamine.

2. Mepyramine, 405C49, and 295C51 greatly delay the onset and retard the progress of the anaphylactic response of isolated guinea-pig ileum to egg albumen at concentrations comparable to those which block the action of added histamine. Larger concentrations are necessary to limit the final extent of the spasm, the amounts causing 50% inhibition varying in different pigs between wide limits (10^{-5} to 3×10^{-7}). The anaphylactic contraction is eliminated only by very high concentrations, sufficient to antagonize the action of spasmogens other than histamine.

3. 405C49 and 295C51, especially the latter, afford greater protection than mepyramine against anaphylactic shock in the intact guinea-pig, but no single dose has given complete protection. The onset of fatal emphysema of the lung can be greatly delayed by these antihistamines, but it frequently develops later unless large doses are used. The majority of animals die, after considerable delay, whatever the antihistamine treatment, other than large doses of 295C51. Vascular congestion of the intestine and other tissues is then a more marked feature than emphysema. These observations are discussed in relation to the role of histamine and other factors in the anaphylactic response.

The author is indebted to Dr. A. C. White for valuable help and criticism ; to Mr. J. A. Calnan, Miss I. A. Carter, and Mrs. E. P. Penson, who carried out most of the experimental work ; and to Mr. J. B. Attlesey for the statistical examination of results.

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THE PROTECTIVE EFFECT OF SYMPATHOMIMETIC AMINES AND OF AMINOPHYLLINE IN THE ANAPHYLACTIC MICROSHOCK OF THE GUINEA-PIG

BY

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Recently we have attempted to assay the protective effect of antihistamines in the anaphylactic shock of the guinea-pig (Armitage, Herxheimer, and Rosa, 1952). In this paper we shall deal with the effect of a number of sympathomimetic amines and of aminophylline.

METHOD

The method has been described earlier (Herxheimer, 1952), and the procedure in the present experiments is similar to that described in our study of the antihistamine effect.

The substances used were adrenaline, noradrenaline, isoprenaline (N-*iso*propylnoradrenaline), ephedrine, methylephedrine, cobefrin (corbasil), neosuprel, and aminophylline. Neosuprel (WIN 3046) is a derivative of isoprenaline in which an α-H-atom has been replaced by an ethyl group:

Methylephedrine is the N-methyl derivative of ephedrine, so that it contains a dimethylamino group.

All substances except the aminophylline were given intramuscularly; aminophylline was given intraperitoneally, as its intramuscular injection tends to cause necrosis. All except ephedrine and methylephedrine were given 15 min. before exposure to shock; ephedrine and methylephedrine were given one hour beforehand. The interval of 15 min. was chosen because the maximum effect of a subcutaneous or intramuscular injection of these substances in man can be expected at about that time. With ephedrine a longer interval was preferred, as oral ephedrine usually takes almost an hour until it acts and as its action may be delayed after injection (Starr *et al.*, 1937). All dosages of 10 mg./kg. or more of ephedrine and methylephedrine caused ruffling of the hair and, in the higher doses, tremor. These symptoms started in some animals 15 min. after injection and lasted for several hours. Isoprenaline 0.2 mg./kg. caused death in two of the eight animals used; with 0.1 mg./kg. one death occurred in 16 animals. Adrenaline 0.2 mg./kg. caused tremor. No other toxic effects were observed.

The statistical methods applied in the present study differ in two respects from those described by Armitage *et al.* (1952). First, the method previously used for measuring the protective effect of each substance occasionally resulted in very large negative responses, when an individual animal happened to show a shorter pre-convulsion time under treatment than in its control observations. To avoid this difficulty we now define the response as follows:

$$y = 100(1 - C/T), \text{ if T is greater than C;}$$
$$y = -100(1 - T/C), \text{ if T is less than C;}$$

where C is the control pre-convulsion time (mean of 2 controls), T the drug pre-convulsion time in seconds. This method differs from that used previously only for negative responses, and the effect on the dose-response relationships will be negligible except possibly at the doses giving very low protection.

Secondly, in order to make the procedure of fitting logistic curves to the dose-response relationships as objective as possible, we have estimated the upper limits of the curves from the data by statistical methods rather than by a preliminary guess.

Some of the substances were tested at too small a number of doses to permit curves to be fitted with any useful degree of accuracy. Logistic curves were fitted for ephedrine, methylephedrine, isoprenaline, and neosuprel. The mean response at 15 mg./kg. ephedrine, and that in one of the experiments with 0.1 mg./kg. isoprenaline, differed widely from the responses at adjacent doses, and have been omitted in the construction of the curves because of the possibility of a technical error. As the responses of ephedrine lay close to those of methylephedrine, the curves of these 2 substances were so fitted that they differed by a constant log-dose interval. This corresponds on the graph to a constant horizontal distance. The results for aminophylline, the highest dose of which (100 mg./kg.) produced complete protection in all four animals, cannot be fitted satisfactorily by a logistic curve. The shape of the dose-response relationship

at high doses of aminophylline is uncertain, and a freehand curve (shown by a dotted line in Fig. 1) has been drawn.

Results

The detailed results are shown in Table I and Fig. 1. It can be seen that all the substances investigated had a protective effect, with the exception of cobefrin. The dose-response curve appears, for those compounds of which a sufficient number of doses have been tried, to have a sigmoid shape. Only aminophylline achieved full protection. Isoprenaline and neosuprel were, weight for weight, the strongest of the amines, followed by adrenaline and noradrenaline. Ephedrine reached approximately the same maximum protective effect as noradrenaline, although with a much higher dosage. Methylephedrine was more effective in very high doses which were not tried with ephedrine. Because of the toxic side-effects of the latter, we have refrained from giving doses higher than 60 mg./kg. It does not seem advisable to use dosages which cause obvious toxic side-effects. We have had some fatalities with 0.1 and 0.2 mg./kg. of isoprenaline and a considerable amount of tremor with 60 mg./kg. of ephedrine. The possibility cannot be excluded that such toxic side-effects influence the usual action of a drug in a decisive way.

Table I

PERCENTAGE PROTECTION UNDER TREATMENT, MEASURED BY INCREASE OF PRECONVULSION TIME (See Method)

Substance	Dose (mg./kg.)	Log Dose	No. of Animals	Mean Protection ± Standard Error	Estimated Protection from Fitted Curve
Adrenaline	0·005	−2·30	7	5·3±14·7	
	0·02	−1·70	6	28·7±12·2	
	0·2	−0·70	7	57·1± 5·8	
Aminophylline	5·0	0·70	6	8·3± 2·7	
	10·0	1·00	6	11·8±10·4	
	20·0	1·30	7	39·4± 9·6	
	40·0	1·60	5	56·0±13·3	
	100·0	2·00	4	100·0	
Cobefrin ..	0·005	−2·30	5	36·2±18·7	
			4	2·0±12·1	
			4	4·8±28·1	
	0·02	−1·70	5	9·2±12·7	
	0·2	−0·70	5	−1·6± 5·1	
Ephedrine	4·0	0·60	7	2·0± 7·8	8
	10·0	1·00	6	20·8±10·2	20
	15·0	1·18	7	*−24·0± 7·7	27
	20·0	1·30	7	42·0±11·0	35
	30·0	1·48	4	48·8± 4·5	44
			4	18·8±11·4	
			6	47·2± 8·5	
	40·0	1·60	6	54·8± 7·1	49
	60·0	1·78	7	31·3±16·3	56
			5	52·2± 3·8	
Isoprenaline	0·001	−3·00	7	14·4±13·0	8
	0·005	−2·30	4	52·0± 7·9	53
			3	39·0±21·6	
	0·02	−1·70	3	46·0±15·2	69
			7	78·3± 5·4	
			5	*38·6± 3·5	
	0·1	−1·00	3	85·3±14·7	70
			7	68·4± 5·0	
	0·2	−0·70	3	61·0±11·8	70
			3	76·3±16·3	
Methylephedrine	10·0	1·00	6	−5·2±10·5	7
	30·0	1·48	6	43·2± 3·6	40
	60·0	1·78	5	47·0± 6·1	53
	100·0	2·00	4	48·5±14·5	60
	160·0	2·20	6	62·2± 2·9	64
	200·0	2·30	6	72·3± 3·8	66
Neosuprel	0·005	−2·30	7	16·4± 8·9	0
			7	18·1±10·6	
	0·02	−1·70	6	4·7±14·9	20
			5	28·6±11·5	
	0·06	−1·22	4	73·8± 7·7	77
	0·1	−1·00	7	80·1± 1·9	78
	0·2	−0·70	7	77·0± 2·4	79
Noradrenaline	0·02	−1·70	4	20·2±12·1	
			5	9·2± 9·2	
	0·2	−0·70	6	18·8±10·0	
	0·4	−0·40	6	31·3±12·1	
			5	36·6± 8·8	
	1·0	0	6	55·7± 3·5	

* These experiments have been omitted in the construction of the graph.

Discussion

If the actions of the various substances are compared, the protective effect of aminophylline appears clearly as the strongest. Full protection is not achieved by any of the sympathomimetic amines, nor by any antihistamine (Armitage *et al.*, 1952). The amount of aminophylline necessary for full protection (100 mg./kg.) is greater than that used with the other substances except methylephedrine, and, theoretically, it seems possible that a similar amount of an adrenaline-like substance would also have given full protection had its action not been too toxic to be tolerated. It is interesting to note that in human asthma aminophylline, given intravenously, is probably the most reliable drug for cutting off acute attacks or relieving the asthmatic state. It is more reliable than the sympathomimetics, as tolerance to it is not so easily acquired and its action is less fleeting. The amount used for intravenous injection in man is 4–8 mg./kg.

The differences in the protective action of the adrenaline-like substances are of a similar order to that found in human asthma. In our experiments isoprenaline and neosuprel are strongest, followed by adrenaline and noradrenaline. In man isoprenaline has been found stronger than adrenaline by Gay and Long (1949) and Bresnick, Beakey, Levinson, and Segal (1949). This is in accordance with our own (unpublished) clinical observations. Neosuprel has been found by Hershfus *et al.* (1951) to be somewhat weaker than isoprenaline. Data about the action of noradrenaline in man are lacking, but our own spirometric observations on the

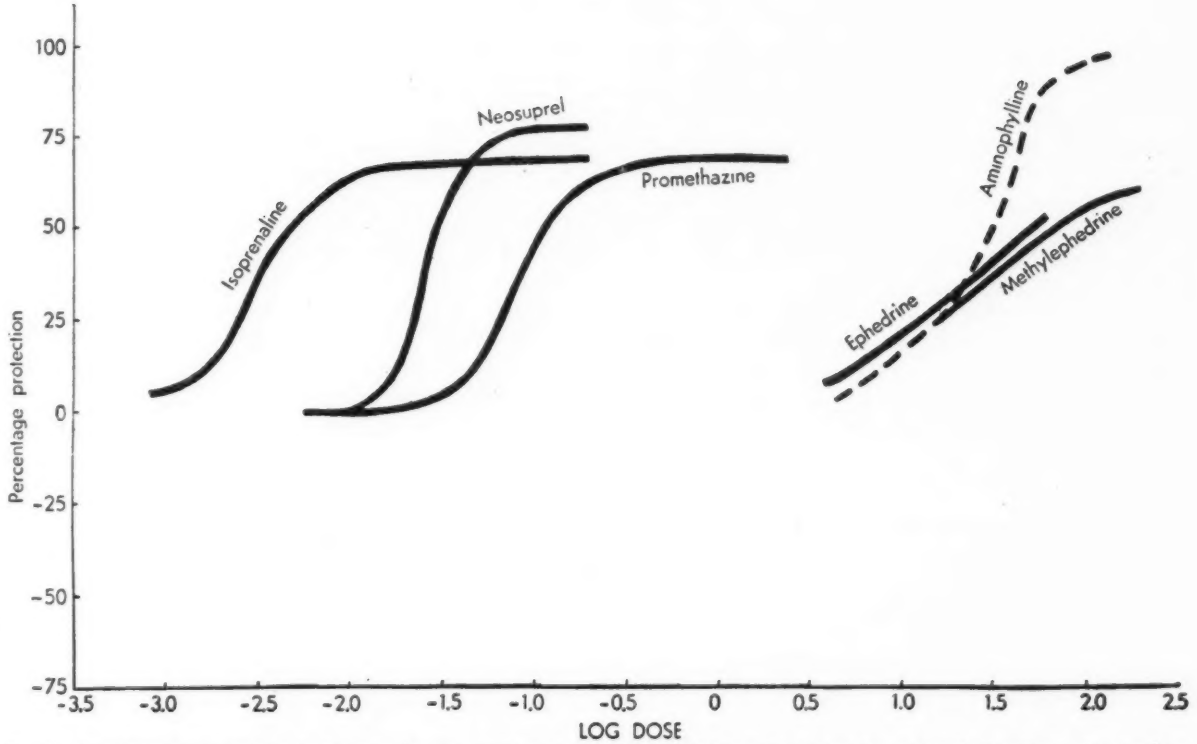

FIG. 1.—Log dose-response curves for isoprenaline, neosuprel, promethazine, ephedrine, methylephedrine, and aminophylline. The log dose has been plotted against the percentage protection given by the dose concerned. Promethazine, one of the stronger antihistamines, has been included for comparison.

increase in vital capacity in the induced asthmatic attack indicate that noradrenaline is considerably weaker than adrenaline (Herxheimer, unpublished). It is remarkable that the order of efficiency is similar in the guinea-pig and in man, although the relative dosage used in the former is very much greater and would be toxic in man.

The protective effect of maximum doses of isoprenaline and neosuprel appears similar to that of the stronger group of antihistamines, whilst maximum doses of adrenaline and noradrenaline appear to reach only the effect of the weaker group of antihistamines. The lowest doses of adrenaline and noradrenaline giving a protective effect do not differ much from the corresponding doses of the weaker antihistamines. Isoprenaline and neosuprel show a protective effect with 5 μg./kg. which is equalled by mepyramine, tripelennamine, and BW 295/C/51. The comparison between sympathomimetic amines and antihistamines thus does not show the fundamental differences which are seen in human asthma. One of the causes for this may be the considerable difference in dosage in man and guinea-pig. The latter requires 30–100 times more of the sympathomimetic amines than man, whilst with some of the antihistamines the dosage is approximately the same, and with others the differences are small.

Ephedrine and methylephedrine, which do not differ in this respect from the other amines, cause toxic side-effects with a comparatively moderate dosage. As can be seen from the figure, ephedrine appears to have, weight for weight, the same protective action as methylephedrine, but the animals tolerate a greater amount of the latter. In human asthma we have found that a double to fourfold dose of methylephedrine is required if ephedrine is to be replaced. In this connection it should be kept in mind that the close similarity of the fitted curves for ephedrine and methylephedrine does not necessarily represent the true relation of the dose-responses of these drugs. As can be seen from Table I, there is a considerable scatter of points, and it appears possible to fit curves representing a greater difference and showing ephedrine, weight for weight, 2–3 times stronger than methylephedrine. Many more experiments would be required to state this relation in more accurate terms.

Cobefrin has not shown any protective action. As far as we know, it has not been tried in human asthma.

Summary

1. The protective effect of aminophylline, adrenaline, noradrenaline, isoprenaline, neosuprel, cobefrin, ephedrine, and methylephedrine in mild anaphylactic shock of the guinea-pig has been investigated.

2. Aminophylline is the only substance with which full protection can be achieved. The other substances achieve, in non-toxic doses, partial protection only. Cobefrin does not protect.

3. The order of efficiency of these substances is approximately the same as that observed in human asthma.

4. The dose-response curve in some of these substances has, as in most antihistamines, a sigmoid shape.

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SOME OBSERVATIONS CONCERNING THE MODE OF ACTION OF THE ANTIHISTAMINIC DRUG "LERGIGAN" (N(α-METHYL-β-DIMETHYL AMINOETHYL)PHENOTHIAZINE HYDROCHLORIDE) IN MOTION SICKNESS

BY

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During recent years many reports have been published concerning the beneficial action of certain antihistamine drugs in the treatment and prevention of motion sickness. Thus Gay and Carliner (1949a, b) found dramamine, the 8-chlorotheophylline salt of diphenhydramine, to be effective against nausea and vomiting in motion sickness. Their findings were confirmed by others. Chinn and Oberst (1950) found benadryl as effective as dramamine in the prevention of air sickness; the best effect, however, was obtained when benadryl was combined with hyoscine. Chen and Ensor (1950) were able to prevent apomorphine-induced emesis in dogs by using benadryl. Engquist (1950, 1951) tested the drug lergigan (Halpern and Schmiterlöw, unpublished) against sea sickness with good results. Chinn, Waltrip, and Massengale (1951) and Chinn, Handford, Cone, and Smith (1952) tested different drugs against sea sickness and found lergigan to be one of the most effective drugs in this respect.

The question naturally arises why antihistamine drugs exert this protective action against motion sickness. The effect is obviously not due to the histamine antagonizing action, since not all antihistamine drugs exert this effect (see Chinn *et al.*, 1952). It has been suggested (Chinn *et al.*, 1950) that the degree of central anticholinergic action might determine in large part the effectiveness of the drug. This suggestion is of great interest when compared with the findings of Johns and Himwich (1950). These authors made intracarotid injections of DFP on animals, and observed that benadryl, dramamine, and lergigan could cure the DFP-induced cortical seizures. Other antihistamines such as thephorin, antistine, pyribenzamine, etc., were without effect. They stated that the curative ability of certain antihistamine drugs in DFP-induced seizures depended upon a cerebral action of these drugs and might be related to an atropine-like quality. It has also been argued that the sedative effect of some antihistamine drugs might be the explanation of their action against motion sickness. Bjerver and Goldberg (1951), using the flicker test and the blink reflex test, showed that a number of antihistamines exerted a central depressant action, and they also studied the effect of these drugs in experimentally induced motion sickness.

It is well known that the blood pressure often falls in motion sickness. If the blood pressure is raised, for example by infusion of dextran, the symptoms may disappear (Engquist, 1951). It is but natural to connect this phenomenon with the early observations of Spiegel and Démétriades (1922) that not only caloric and galvanic stimulation of the labyrinth but also rotatory stimulation cause a fall of blood pressure. The mechanism behind this blood pressure fall seems similar to the reflex effect caused by stimulation of the vagal depressor nerves.

The present investigation was performed in order to find out how the antihistaminic drug lergigan (Recip, Co., Stockholm), known to be active against motion sickness, would influence the blood pressure fall obtained when stimulating the eighth cranial nerve in cats.

METHODS

Electrical Stimulation of the Eighth Cranial Nerve.—Cats have been used throughout the experiments. Most animals were lightly anaesthetized with allobarbital, 0.03–0.04 g. per kg. body weight given intravenously. In addition some cats were anaesthetized with 0.06 g. chloralose per kg. A tracheal cannula was inserted.

The skin and muscles above the occipital bone were removed. The occipital bone was excised to expose the cerebellum, which was sucked out in order to

make the eighth cranial nerve fully visible between the internal auditory meatus and the medulla oblongata.

The electrode used for stimulation of the eighth nerve consisted of a fine needle, the diameter of which did not exceed 0.05 mm. The insulated electrode, with the tip exposed, was inserted by means of a micromanipulator into the eighth nerve just outside the internal auditory meatus. The stimulating current was generated by thyratron discharges. The electrical stimuli were of rectangular shape, and the pulse duration of the stimulating current was about 5 msec. at the maximal strength used. The frequency of the shocks used was 1–2 per second at intensities of 8–10 volts. After locating a point within the eighth nerve which in response to stimulation gave a maximal fall in blood pressure, recorded with a mercury manometer from the femoral artery, the electrode was left in that position during the experiment.

Injections were made through the external jugular vein. In order to abolish the motor activity of the muscles in response to vestibular stimulation the animals were curarized. Artificial respiration was given.

The Effect of Lergigan on Some Other Autonomic Reflexes.—The effect of lergigan was studied on:

1. The blood pressure rise caused by carotid occlusion. The experiments were carried out on cats under chloralose anaesthesia.
2. The blood pressure fall caused by stimulation of the sinus nerve. Cats under sodium pentobarbitone were used. The right sinus nerve was dissected free and stimulated electrically, using a frequency of 20 per second at an intensity of about 2 volts. The duration of each stimulus was 1 msec.
3. The blood pressure fall caused by stimulation of the depressor nerve. Rabbits under urethane anaesthesia were used.
4. The blood pressure fall caused by stimulation of the peripheral end of the vagus. Cats under chloralose were used.
5. The effect on respiration caused by electrical stimulation of the central end of the superior laryngeal nerve. Cats under chloralose were used. The cut right superior laryngeal nerve was stimulated at a frequency of 20 per second. Stimulus length 1 msec., intensity about 0.5 volt.

The Influence of Rotation upon the Blood Histamine.—Some experiments were performed in order to find out whether rotation would cause any change in the histamine content of the blood. Rabbits were used for these experiments. They were put in the rotation apparatus described by Gernandt (1949). After rotation the rabbits were immediately killed and the blood collected in acid alcohol. The amount of histamine was then determined according to the method described by Schmiterlöw (1949).

The Action of Lergigan on Gastric Motility.—In order to study the effect of lergigan on gastric motility caused by vagus stimulation the following experiments were performed. Cats under chloralose were used. A rubber balloon was inserted into the stomach and connected with a Marey tambour. The thorax was opened on the right side, artificial respiration being given. After removal of the lower lobes of the right lung an electrode was placed on the peripheral part of one of the cut vagus branches running along the oesophagus. Blood pressure was recorded from one of the carotid arteries.

Results

Stimulation of the Eighth Cranial Nerve.—When the eighth cranial nerve was stimulated in the way described above a fall in blood pressure and a slowing of the heart were regularly observed. This blood pressure fall comes on gradually and persists during the stimulation. Fig. 1 A shows this effect of stimulation. On the same record is seen the effect of varying doses of histamine dihydrochloride, injected intravenously, and also the effect of acetylcholine hydrochloride. When lergigan in a dose of 2 mg./kg. body weight was given intravenously and the same experiment was repeated it was observed that the stimulation of the eighth cranial nerve no longer caused a fall in blood pressure (Fig. 1 B). The effect of histamine was totally abolished, whereas the action of acetylcholine persisted, although markedly impaired. In order to find out whether this blocking of the effect of histamine was of any importance in the mechanism which enabled lergigan to block the blood pressure fall, caused by stimulation of the eighth cranial nerve, the following experiment was performed. After stimulation of the eighth cranial nerve the effects of acetylcholine and histamine were studied (Fig. 2 A), and atropine (0.3 mg./kg.) was then given. After atropine the

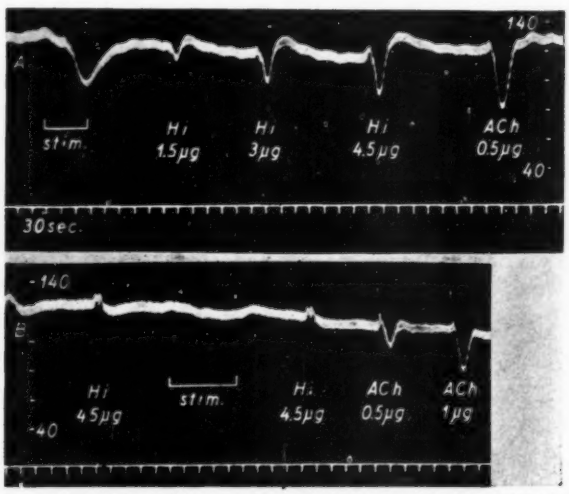

FIG. 1.—Cat (allobarbital). Blood pressure record. Stim.=electrical stimulation of eighth cranial nerve. Hi=histamine dihydrochloride. ACh=acetylcholine hydrochloride. Between A and B 2 mg./kg. lergigan intravenously.

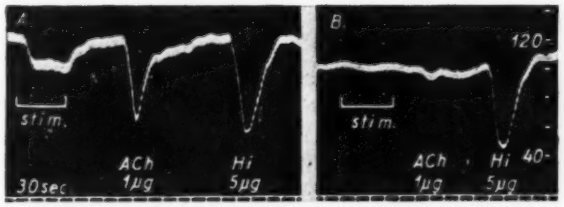

FIG. 2.—Cat (allobarbital). Blood pressure record. Stim.=electrical stimulation of the eighth cranial nerve. Hi=histamine dihydrochloride. ACh=acetylcholine hydrochloride. Between A and B 0.3 mg./kg. atropine sulphate intravenously.

stimulation of the eighth nerve does not lead to any fall in blood pressure, seen in Fig. 2 B, where it can also be observed that the effect of histamine was not influenced by this small dose of atropine. Obviously it is not the histamine antagonizing power of lergigan which is responsible for the effect on the stimulation of the eighth cranial nerve.

If the effects of lergigan and atropine were compared it was noted that the ability of lergigan to block the blood pressure fall induced by stimulation of the eighth cranial nerve was very constant, whereas atropine in the dose used (0.3 mg./kg.) did not always give a complete blocking effect. If, however, both vagi were cut the effect on the blood pressure totally disappeared.

If the stimulation of the eighth cranial nerve was performed in cats under chloralose anaesthesia, and allobarbital (30 mg./kg.) was given, no obvious change in the effect could be observed (Fig. 3). These experiments were done in order to see whether an increase in central depression would have any effect.

The Effect of Lergigan on Other Autonomic Reflexes.—From the experiments cited above it is not clear whether the action of lergigan should be regarded as a central or peripheral action. In order to elucidate this question the experiments on other reflexes, mentioned above (see Methods), were carried out.

1. Clamping both carotids in a cat under chloralose leads to a marked rise in systemic blood pressure.

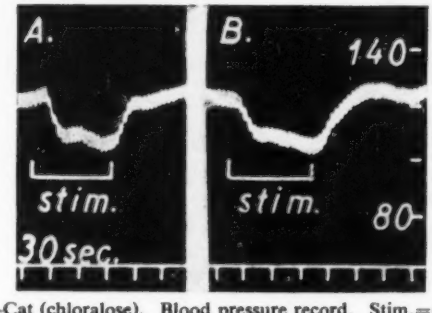

FIG. 3.—Cat (chloralose). Blood pressure record. Stim.=electrical stimulation of the eighth cranial nerve. Between A and B 30 mg./kg. allobarbital intravenously.

This effect is not influenced by lergigan, as can be seen in Fig. 4.

2. If the sinus nerve is stimulated electrically the result on the blood pressure is different for different anaesthetics. Neil, Redwood, and Schweitzer (1949) thus showed that when the sinus nerve is stimulated in cats under pentobarbitone anaesthesia the result is invariably a fall in blood pressure. This observation could easily be confirmed; stimulating the right sinus nerve electrically in cats under pentobarbitone caused a slight but distinct lowering of the blood pressure. This effect was not influenced by lergigan (Fig. 5). In this figure respiration was also registered from the tracheal cannula.

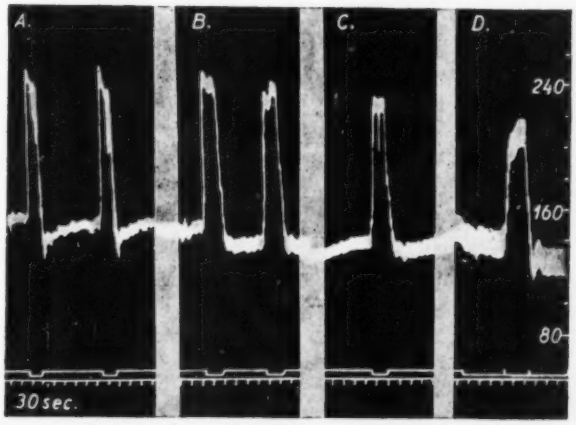

FIG. 4.—Cat (chloralose). Blood pressure record. Blood pressure recorded from femoral artery. Signal indicates clamping of both carotids. A before lergigan, B after 2 mg./kg. lergigan, C and D after 4 mg./kg. lergigan intravenously. In D the cat was allowed to breathe pure oxygen.

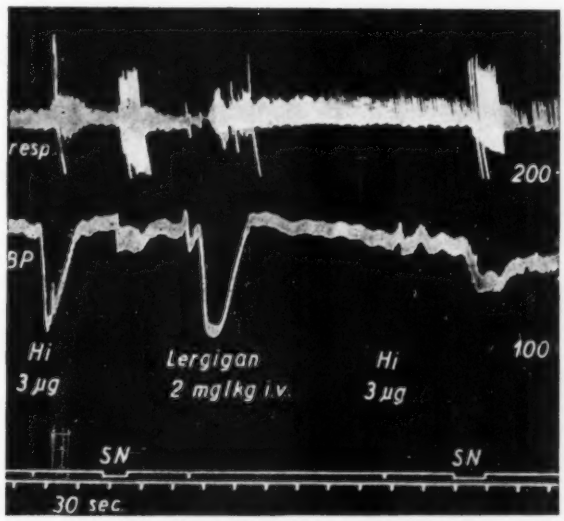

FIG. 5.—Cat (chloralose). Respiration recorded from tracheal cannula (upper curve). Blood pressure record (lower curve). Signal indicates electrical stimulation of the right sinus nerve (SN). Hi=histamine dihydrochloride. The effect of stimulation of the sinus nerve is seen before and after 2 mg./kg. lergigan intravenously.

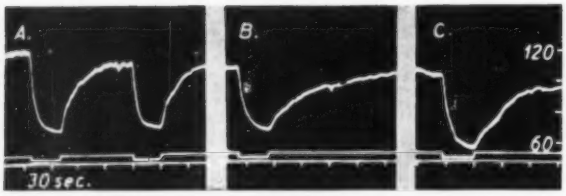

FIG. 6.—Rabbit (urethane). Blood pressure record. Signal indicates electrical stimulation of the right depressor nerve. A before lergigan, B after 2 mg./kg., and C after 4 mg./kg. lergigan intravenously.

3. In the rabbit the depressor nerve can easily be dissected free from the vagus trunk and thus be stimulated separately. The response to such stimulation, a blood pressure fall, was not influenced by lergigan (Fig. 6).

4. So far the experiments have not shown any sign of a central blocking action of lergigan. It would be of interest to see whether lergigan exerts any peripheral action which could explain the ability of the substance to abolish the blood pressure fall caused by the stimulation of the eighth cranial nerve. For that reason the peripheral end of the cut right vagus was stimulated electrically, using such stimulus as would cause a marked blood pressure fall and bradycardia. When lergigan was then given this effect was almost totally abolished (see Figs. 7 A and B). In the same figure it can be noted that the effect of acetylcholine is only partly blocked by the dose of lergigan used, whereas the electrical vagus stimulation is almost completely blocked.

5. Figs. 7 A and B also show that the effect on the respiration produced through stimulation of the right superior laryngeal nerve is not influenced by lergigan.

The Blood Histamine Level in Rabbits After Rotation.—The rabbits were rotated (angular stimulation) for different lengths of time, 10, 15, 25, and 30 minutes. The animals were killed immediately after the rotation had stopped and the blood was collected. When the amount of histamine in the blood was estimated no significant difference from the normal histamine level in rabbits was found. Neither was there any difference between blood from rabbits that had been rotated for different lengths of time.

The Action of Lergigan on Gastric Motility.—When stimulating vagus branches below the heart strong contractions of the stomach were observed. After lergigan these contractions were markedly reduced, as can be seen from Fig. 8.

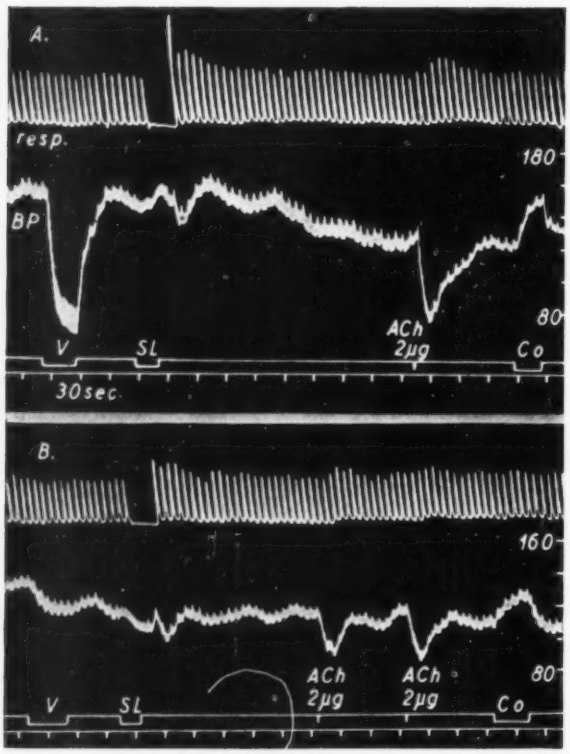

FIG. 7.—Cat (chloralose). Respiration recorded from tracheal cannula (upper curve). Blood pressure record (lower curve). Signal indicates electrical stimulation of the cut peripheral vagus (V) and of central end of the cut superior laryngeal nerve (SL) and clamping of right carotid (Co). ACh = acetylcholine hydrochloride. A before, B after, 2 mg./kg. lergigan intravenously.

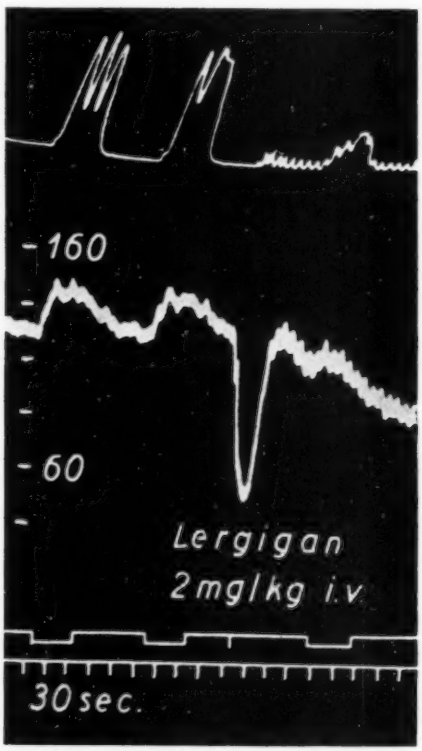

FIG. 8.—Cat (chloralose). Motility of the stomach (upper curve). Blood pressure record (lower curve). Signal indicates electrical stimulation of the cut peripheral vagus below the heart. Artificial respiration. The effect of stimulation is seen twice before and after 2 mg./kg. lergigan intravenously.

Discussion

The experiments described here have shown that the antihistamine drug lergigan is able to prevent in anaesthetized cats the lowering of the blood pressure induced by electrical stimulation of the eighth cranial nerve.

It appears reasonable to assume that the ability of such a drug as lergigan to prevent or cure motion sickness at least to some extent is due to this prevention of the blood pressure fall which is a common symptom in motion sickness. If such drugs as would raise the blood pressure are given to persons suffering from motion sickness, the nausea and vomiting disappear (Engquist, 1951). It is but natural to think that a beneficial effect will be obtained with a drug which will prevent the lowering of the blood pressure.

Lergigan also blocks the effect of vagus stimulation on the stomach. This fact will also offer an explanation to the ability of lergigan to prevent or cure motion sickness.

However, one must take into account the fact that lergigan also exerts a sedative effect (see Bjerver and Goldberg, 1951), which might certainly be of importance in the prophylaxis and treatment of motion sickness in man.

There still remains to answer the question in which way lergigan (and other antihistamine drugs, reported in the literature to be of value against motion sickness) exerts this effect to prevent a blood pressure fall.

It is obvious that this effect has nothing to do with the histamine antagonizing action of the drugs in question. This is clear from several facts. It has been shown by other authors that only some of the antihistamines exert this beneficial action against motion sickness, and amongst those are not the most specific histamine antagonizing drugs. Our experiments also show that rotation does not increase the blood histamine level. And, finally, even if the effect of histamine is not counteracted it is possible by using atropine to block this blood pressure fall.

If the action was on the central nervous system, as suggested by, e.g., Chinn *et al.* (1950), it seems natural that also other autonomic reflexes would be abolished by lergigan. This is, however, not the case, as is shown in our experiments. The stimulation of the central part of the sinus nerve, for example in cats under chloralose, leads to a blood pressure fall which is not abolished by lergigan. The carotid occlusion test is not affected by lergigan. The blood pressure fall following stimulation of the depressor nerve is not counteracted by lergigan. The effect of stimulation of the central end of the superior laryngeal nerve is not influenced by lergigan.

Lergigan is able to prevent the blood pressure fall caused by stimulation of the peripheral end of the vagus. It seems natural to assume that this effect is similar to its ability to block the blood pressure fall when the eighth cranial nerve is stimulated. The blood pressure fall caused by this stimulation is very likely brought about by an activation of the vagus centre, an assumption which is supported by the fact that if both vagi are cut the stimulation of the eighth cranial nerve no longer leads to a fall in blood pressure. Both atropine and lergigan are able to prevent the blood pressure fall following upon electrical stimulation of the eighth cranial nerve. It is thus obvious that the effect of lergigan in this respect is due to a blocking of the peripheral vagus transmission. This view is supported by the fact that lergigan has a very strong anti-acetylcholine action.

It was assumed by Spiegel (1926) that the blood pressure fall induced by stimulation of the eighth cranial nerve was due to a mechanism similar to that observed when the depressor nerve is stimulated. This does not, however, seem very likely from the present experiments. The depressor reflex is not influenced by lergigan. The blood pressure fall caused by stimulation of the depressor nerve is, as shown by Folkow, Ström, and Uvnäs (1950) and Celander and Folkow (1951), due to an inhibition of the vasoconstrictor centre leading to a diminished vasoconstrictor outflow. Therefore this reflex will not be influenced by lergigan, since it does not involve the activation of the vagus. When the eighth cranial nerve is stimulated the ensuing blood pressure fall is due to an increased outflow in the vagus, the effect of which can be blocked by lergigan. The activation of the vagus seems to be rather natural when it is taken into account that vestibular fibres are distributed to the reticular formation of the medulla oblongata where they, *inter alia*, appear to reach the vagus and glossopharyngeal nuclei. This would be in accordance with the occurrence of vomiting and cardiovascular changes observed in vestibular stimulation.

From our experiments we think it is justified to draw the conclusion that the beneficial action exerted by lergigan and certain other antihistamines on motion sickness is partly due to their ability to block the activity of the vagus, thus preventing a blood pressure fall and also inhibiting gastric motility, etc. This effect is due to an atropine-like, peripheral action. In this connexion it should be pointed out that the antihistamines which have been used against motion sickness have in common

that they are fairly non-specific antihistamines, e.g., they do not only antagonize histamine, but they also counteract the effect of acetylcholine. It must, however, also be remembered that antihistamines usually have a sedative action which certainly plays a role in the prevention of motion sickness. This fact could be illustrated by the well-known superiority of hyoscine over atropine as regards prevention of motion sickness.

Summary

1. The eighth cranial nerve has been stimulated electrically in cats. The result is a fall of blood pressure.

2. This blood pressure fall is abolished by moderate doses of the antihistamine lergigan and by atropine.

3. Lergigan does not interfere with the central part of other autonomic reflexes.

4. Lergigan blocks the effect of peripheral vagus stimulation.

5. The blood pressure fall caused by stimulation of the eighth cranial nerve is obviously due to an activation of the vagus centre.

6. The effect of lergigan against motion sickness is partly due to its atropine-like action, which abolishes vagus effects.

7. Angular stimulation does not change the amount of histamine in the blood.

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EXPERIMENTS ON THE SIDE-EFFECTS OF DRUGS

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The need to study drugs in a way which would allow unbiased analysis of their side-effects arose from the fact that some symptoms of motion sickness closely resemble the side-actions of motion sickness remedies (Glaser and Hervey, 1951). Such an investigation, moreover, could be expected to have fundamental implications and indicate new ways in which drugs could be assessed.

Materials and Methods

Subjects.—52 Asian medical students and 20 European members of the British Army took part in this investigation. Four medical students and 10 members of the Army were women. The subjects' ages were between 18 and 31 years. There was no self-selection among the medical students, because an entire class volunteered for this experiment, but the Service men and women volunteered following a request sent to certain troops stationed in Singapore, and they were further selected on account of their doing duties from which they could be spared.

Procedure.—Two separate experiments were performed.

In the first experiment the medical students were given single doses of drugs or a dummy at 7-day intervals. They all took, in turn, 1 mg. of *l*-hyoscine hydrobromide, 25 mg. of promethazine hydrochloride (" phenergan "), 25 mg. of diphenhydramine hydrochloride (" benadryl "), and a dummy, but the order of issuing varied, so that each possible sequence was represented in two or three subjects. The drugs and dummy were indistinguishable and contained identical amounts of colouring, flavouring, and lactose excipient. They were labelled and issued according to a code the key to which was not available until after the results had been worked out, and they were handed out in envelopes marked with the date and the subjects' names. This made it possible to keep the subjects in complete ignorance of the number or types of substances involved. The drugs and dummy were always taken at the same time of the day, ½ hour after breakfast, and during the next 3 hours the subjects' activities were controlled and sedentary. One and a half hours and 3 hours after taking the drugs the subjects were given identical forms in which questions were put in such a way that the presence of symptoms sometimes required an affirmative reply and sometimes a negative one and in which answers could be provided by underlining single words. In order to ensure that no symptoms were missed there was an additional question asking the subjects to describe what were, in their opinions, the effects of the substances taken. The subjects were asked not to rely on memory, but to note their symptoms at the time of observation, even if they themselves considered these symptoms imagined or irrelevant, and they were also asked to record their own pulse rates. At the end of 12 hours they filled in a third and less elaborate questionnaire in which they were asked to record their symptoms after the first 3 hours and in which some scope was given to their recollections of how they had felt during the latter 9 hours. In order to acquaint the subjects with the questionnaires and accustom them to the experiment a preliminary trial was staged under identical conditions, except that no substances were issued.

In the second experiment the members of the Services were tested in two batches, one of 8 and one of 12 subjects, and they were given drugs or a dummy three times daily (in the early morning, at noon, and late in the afternoon) for two 6-day periods separated by one day on which there were no experimental procedures. On the first and last day of each 6-day period dummies were given to all subjects. On the second to fifth day of each period the subjects were given hyoscine or promethazine: half the subjects in each batch took hyoscine during the first week and promethazine during the second, while the other half took these drugs in the reverse order. Hyoscine hydrobromide was given in an initial dose of 1 mg. in the morning of the second day, followed by ½ mg. three times daily, so that 2 mg. was given on the first day and 1½ mg. on subsequent days. Promethazine was given in doses of 25 mg. in the morning and afternoon of the second day, followed by 25 mg. each morning; dummies were given to these subjects at times when medication was not required, so that they continued to take a substance at set hours 3 times daily. This schedule made it necessary to keep the drugs and dummy in correctly labelled containers, but the substances were made up as before and they were again issued in envelopes marked with the subject's name, the date, and the time. Questionnaires were completed every noon and evening, and precautions taken were the same as in the other experiment, so that the subjects could not know what they were taking and what responses were expected.

Discussion of Drugs and Dosage.—The drugs given were chosen because they are probably the most effective preventives of motion sickness. Claims have been made in favour of the 8-chloro-theophyllinates of promethazine ("avomine") and of diphenhydramine ("dimenhydrinate," "dramamine"), but there is reason to believe that the effects of these drugs on motion sickness are the same as those of their hydrochlorides which were given in the present experiment. (The evidence has been reviewed by Glaser and Hervey, 1951.)

The single doses given to the medical students were the same as those given in a previous experiment at sea, and the reasons for choosing these amounts have been stated (Glaser and Hervey, 1951). The dose of diphenhydramine was perhaps somewhat small, but by no means excessively small (Hervey, 1952). In the second experiment the subjects were not available for long enough to test more than two drugs, and it was decided to omit diphenhydramine, which is probably less effective as a motion sickness preventive than hyoscine or promethazine (Strickland, Hahn, and Adler, 1950; Glaser and Hervey, 1951), and which may be inconvenient for continued treatment owing to the short duration of its action (Landau, Marriott, and Gay, 1948). The dosage in this experiment was chosen for a number of reasons. Hyoscine has been used as a seasickness remedy in doses of 2 mg. in 24 hours (Glaser, 1952), but it was feared that prolonged treatment would cause cumulative effects. Fifty mg. of promethazine was considered suitable for the first day, but the action of promethazine is so prolonged that cumulative effects seemed likely even with single daily doses (Bain, Broadbent, and Warin, 1949). Moreover, many people adapt themselves to motion after 24 hours, and nearly all after four days. Thus, in the treatment of motion sickness, a reduction of dosage would be desirable after the first day, both on grounds of cumulative effects and of adaptation to motion, while treatment could usually cease after four days.

Statistical Calculations.—The associations between the frequencies of symptoms were worked out by 2×2 tables, applying Yates's correction for continuity and making use of the χ^2 method.

Results

Effects of Single Doses During the First Three Hours.—Owing to various unavoidable causes only 49 students took part in all tests. The results are given in Table I, and statistically significant differences are shown in Table II. No additional infor-

TABLE I

EFFECTS OF SINGLE DOSES. INCIDENCE OF SYMPTOMS DURING THE FIRST 3 HOURS

Symptom	No. of Subjects Showing Symptom				
	No Treatment (Initial Test)	Dummy	Hyoscine 1 mg.	Promethazine 25 mg.	Diphenhydramine 25 mg.
Asleep ..	—	—	7	4	1
Drowsiness ..	22	14	41	35	28
Fatigue ..	22	12	30	35	24
Inability to concentrate ..	4	5	14	12	6
Excitement ..	—	—	3	1	—
Headache ..	5	10	22	15	8
Nausea ..	3	10	23	15	8
Giddiness ..	2	2	26	8	5
Blurred vision	—	2	13	4	4
Ringing ears..	—	4	4	1	—
Flushing of face	—	1	11	—	2
Dryness of mouth ..	14	9	46	14	10
Considers drug pleasant ..	—	3	5	3	3
Considers drug unpleasant	—	4	33	17	11
Total showing any effect ..	42	29	48	43	35
Total receiving treatment ..	52	50	50	50	49

TABLE II

STATISTICAL SIGNIFICANCE OF DIFFERENCES GIVEN IN TABLE I

Only significant differences are given ($p<0.05$). Highly significant differences ($p<0.01$) are marked *

Substances Compared	Symptom	χ^2
Hyoscine—promethazine	Giddiness	12·879*
	Blurred vision	4·536
	Dryness of mouth	40·042*
	Drug considered unpleasant	9·000*
Hyoscine—diphenhydramine	Drowsiness	6·111
	Headache	7·711*
	Nausea	8·799*
	Giddiness	18·204*
	Blurred vision	4·353
	Flushing of face	5·660
	Dryness of mouth	48·753*
	Drug considered unpleasant	17·287*
	Total showing effect	9·288*
Hyoscine—dummy	Drowsiness	27·313*
	Fatigue	11·864*
	Inability to concentrate	4·159
	Headache	5·561
	Nausea	6·605
	Giddiness	26·243*
	Blurred vision	7·951*
	Flushing of face	7·671*
	Dryness of mouth	52·364*
	Drug considered unpleasant	33·633*
	Total showing effect	18·295*
Promethazine—dummy	Drowsiness	16·006*
	Fatigue	19·430*
	Drug considered unpleasant	8·681*
	Total showing effect	8·383*
Diphenhydramine—dummy	Drowsiness	7·453*
	Fatigue	5·637

mation could be obtained by a separate study of the questionnaires completed after one and a half and after three hours (see below), and Table I gives the incidence of symptoms during the first three hours, irrespective of the fact whether a subject reported any particular symptom once or twice during that time.

Drowsiness and fatigue were about equally frequent after hyoscine and promethazine, and significantly more frequent after any drug than after the dummy. Apart from these symptoms, however, hyoscine caused the most side-effects. Thus, after taking hyoscine, over 90% of the subjects had dry mouths, about half the subjects felt giddy, and about a quarter of the subjects complained of blurred vision or flushing of the face, and all these symptoms were significantly less frequent after taking any of the other substances. Moreover, hyoscine was considered unpleasant by a significantly larger number of subjects than any other substance. Inability to concentrate was about equally frequent after hyoscine and promethazine; the difference between hyoscine and the dummy, however, was significant, while the difference between promethazine and the dummy was not statistically significant.

The only apparent discrepancy between questionnaires filled in after one and a half hours and those completed after three hours was that drowsiness and fatigue were more frequent three hours than one and a half hours after taking promethazine, but it was found that this difference was not statistically significant ($\chi^2=3.248$; $p>0.05$).

It is interesting that symptoms were not confined to those included in the questionnaires. Thus, a question was asked about excitement, but only four positive replies were received, three of which were recorded after hyoscine, the only substance likely to cause excitement. No question was asked about flushing of the face, but this symptom was recorded 14 times, 11 of which were after hyoscine.

The average pulse rate was 75 after hyoscine and 80 after the other substances. The difference between hyoscine and the dummy was statistically significant ($t=2.305$; $p<0.05$). On the preliminary day when no drugs were given the average pulse rate was 82.

Table I and Fig. 1 show that symptoms were frequent during the initial test in which no drugs were given. Indeed, significantly more subjects had symptoms on that day than later when they were given dummies ($\chi^2=5.217$; $p<0.05$). When drugs were given, the percentage of subjects who had any symptoms, whatever the treatment, gradually diminished, but there was a sharp rise on the last day (Fig. 1), and significantly more subjects had symptoms on that day than the previous one ($\chi^2=5.742$; $p<0.05$). This does not invalidate the results, because the order of issuing was randomized and an equal number of subjects took each drug on each day, but it has implications which will be discussed below.

Late Effects of Single Doses.—The results are given in Table III, and statistically significant differences are shown in Table IV. Drowsiness was significantly more frequent after promethazine than after any other substance, while dryness of the mouth was significantly more frequent after hyoscine than after the other substances. Giddiness was also the most frequent after hyoscine, but only the difference between hyoscine and the

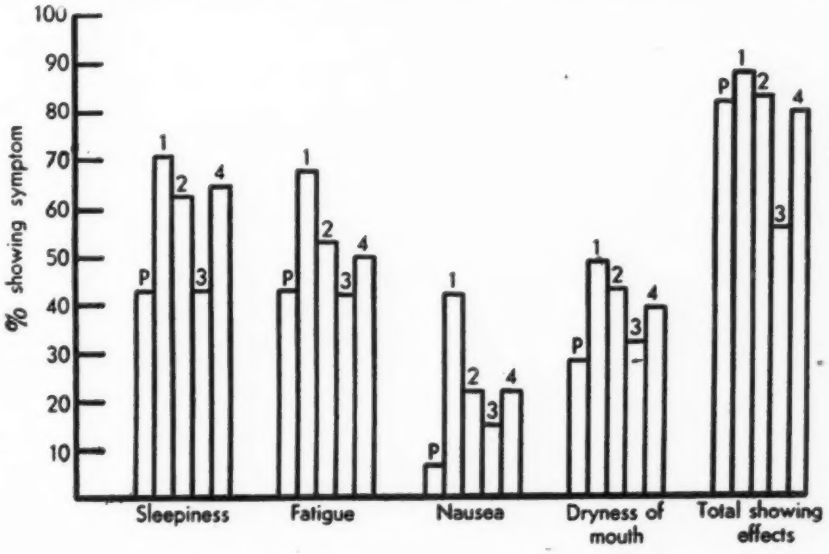

FIG. 1.—Day-to-day incidence of main symptoms during the first 3 hours after taking drugs and dummy. Calculated from all effects recorded on any one day, whatever the treatment. P = preliminary test when no substances were given. Numbers 1–4 indicate successive days when drugs and a dummy were given to similar numbers of subjects.

TABLE III

EFFECTS OF SINGLE DOSES. INCIDENCE OF SYMPTOMS AFTER THE FIRST 3 HOURS

Symptom	No. of Subjects Showing Symptom			
	Dummy	Hyoscine 1 mg.	Promethazine 25 mg.	Diphenhydramine 25 mg.
Drowsiness ..	3	20	32	8
Inability to concentrate	—	2	5	1
Headache	2	9	9	2
Nausea	—	3	1	1
Giddiness	1	10	4	3
Blurred vision ..	—	1	1	1
Flushing of face ..	—	3	1	—
Dryness of mouth .	4	20	3	2
Total showing any effect	6	35	32	14
Total receiving treatment	50	50	50	49

TABLE IV

STATISTICAL SIGNIFICANCE OF DIFFERENCES GIVEN IN TABLE III

Only significant differences are given ($p<0.05$). Highly significant differences ($p<0.01$) are marked *

Substances Compared	Symptom	χ^2
Hyoscine—promethazine	Drowsiness	4·848
	Dryness of mouth	14·457*
Hyoscine—diphenhydramine	Drowsiness	5·720
	Dryness of mouth	16·453*
	Total showing effect	15·375*
Hyoscine—dummy	Drowsiness	14·455*
	Giddiness	6·537
	Dryness of mouth	12·333*
	Total showing effect	32·410*
Promethazine—diphenhydramine	Drowsiness	21·420*
	Total showing effect	11·104*
Promethazine—dummy	Drowsiness	34·462*
	Total showing effect	26·528*

dummy was significant. Nine subjects each had headaches after promethazine and hyoscine, and the differences between these and the other substances just failed to be significant ($\chi^2=3.547$ and 3.682 ; $p>0.05$).

The only apparent difference between diphenhydramine and the dummy was a slightly higher incidence of drowsiness after the former, but it was not statistically significant, and this suggests that the effects of diphenhydramine had mostly disappeared after the first three hours.

Effects of Continued Treatment.—Only 17 out of 20 subjects completed the experiment in which drugs were taken repeatedly for four days. One woman withdrew on the first day when she had taken nothing but dummies, another after taking promethazine for one day, and one man after a single dose of hyoscine hydrobromide. The results obtained from the other 17 subjects are given in Table V. Statistical calculations are given in Table VI. Although two questionnaires were completed by each subject on each day there was no significant difference between the answers, and for this reason symptoms noted on each day are shown as a single entry whether they were recorded by one subject once or twice in the same day. On the first day of this experiment when only dummies were given, 12 subjects (70%) had symptoms, and the results generally conformed with those obtained in the other experiment when no substances were given (Table I). The first day of the second six-day period was taken as a control day, because only dummies were given on this day and there had been no medication for two days beforehand.

TABLE V

EFFECTS OF CONTINUED TREATMENT

(17 subjects receiving each treatment)

Symptom	No. of Subjects Showing Symptom								
	Control Day—Dummies Only*	Hyoscine				Promethazine			
		Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4
Drowsiness ..	3	9	9	8	5	10	5	3	3
Inability to concentrate ..	—	3	1	1	1	—	—	1	—
Excitement ..	1	2	—	1	—	1	—	1	—
Headache ..	4	7	4	5	1	5	4	3	2
Nausea	1	4	1	2	3	1	—	—	—
Blurred vision ..	2	4	5	5	7	2	—	—	—
Flushing of face	2	3	3	7	2	1	2	1	—
Dryness of mouth	3	12	9	5	6	3	1	2	2
Giddiness ..	—	7	3	2	—	2	1	—	1
Total showing effect ..	4	13	13	12	11	12	6	6	5

* See text.

TABLE VI

STATISTICAL SIGNIFICANCE OF DIFFERENCES GIVEN IN TABLE V

Only significant differences are given ($p<0.05$). A highly significant difference ($p<0.01$) is marked *

Substances Compared	Day of Experiment	Symptom	χ^2
Hyoscine .. Dummy ..	1 Control†	Total affected	7·529*
Promethazine .. Dummy ..	1 Control†	Total affected	5·785
Promethazine .. Dummy ..	1 Control†	Drowsiness	4·484
Promethazine .. ,, ..	1 4	Total affected	4·235
,, .. ,, ..	1 4	Drowsiness	4·484

† See text.

Both drugs caused symptoms in a significantly greater number of subjects than the dummy. Drowsiness was about equally frequent after both drugs, but the difference between hyoscine and the dummy was not significant ($\chi^2=3.220$; $p>0.05$). Other symptoms were again more frequent after hyoscine, and this is especially evident if all results obtained during the experiment are considered. Taking the nine symptoms given in Table V, the total number of possible symptoms in four days and with 17 subjects was 612. The number of symptoms recorded after hyoscine was 147, while the number recorded after promethazine was 56, and the difference was significant ($\chi^2=4.835$; $p<0.05$).

Further analysis of the results shows that there was a decline of all symptoms (apart from blurred vision when taking hyoscine) during continued treatment with either drug. With promethazine both the total number affected and the incidence of drowsiness decreased significantly between the first and fourth day of treatment (Table VI), but if all symptoms are taken into consideration this becomes evident for hyoscine also. One hundred and fifty-three symptoms were possible on one day. Fifty-one symptoms were recorded on the first day of taking hyoscine and 25 on the fourth, the difference being highly significant ($\chi^2=10.942$; $p<0.01$). The figures for promethazine were 25 and 8 respectively, and the difference was again highly significant ($\chi^2=8.696$; $p<0.01$). Table V shows that this decrease was gradual and therefore probably caused by habituation, not by a reduction of dosage after the first day of treatment.

Discussion

It can be concluded that hyoscine hydrobromide, promethazine hydrochloride, and diphenhydramine hydrochloride all caused drowsiness and fatigue in a significant number of subjects, while other symptoms were proportionate to the effectiveness of these drugs against seasickness (cf. Glaser and Hervey, 1951). This supports the view that the essential action of antihistamine substances on motion sickness is determined by their hyoscine-like properties, and that side-effects may be inevitable if powerful medication is required (Burn, 1950). It was confirmed that diphenhydramine has a very short action (Landau, Marriott, and Gay, 1948), while promethazine remains effective for a long time (Bain, Broadbent and Warin, 1949). It can be concluded also that fears of cumulative poisoning by hyoscine or promethazine in the present dosage are unfounded and that some tolerance to both hyoscine and promethazine may be acquired within a few days.

Psychological responses were undoubtedly produced by the experimental conditions, but these responses could not have invalidated the results because the experiments were designed to minimize all accidental influences. The evidence of such responses, however, is of great fundamental interest. Symptoms were about equally frequent on the first day of both experiments, whether dummies were given or not, while the giving of drugs and dummies apparently reduced the incidence of effects ; it seems safe to conclude that some symptoms were present because the subjects were asked to look out for them, not because substances had been given. The finding that symptoms gradually decreased not only when drugs were being taken continuously but also when single doses of different substances were given at seven-day intervals suggests that the interest in the experiment must have stimulated certain responses, and this is borne out by the observation that symptoms increased on the last day of the experiment if drugs were given on that day. All this shows a marked similarity with the results of a previous investigation (Glaser and Hervey, 1952) in which 100 men were given varying medications in a random sequence and exposed to identical wave motion six times at 48-hour intervals, and in which there was also a decline of subjective and objective symptoms of seasickness throughout the experiment, followed by an increase on the last day. (In that investigation, as in the present one, the subjects knew nothing about the experimental design, but expected to be told about it after the last test.) The conclusions appear justified that (1) results obtained from human subjects in single tests are liable to fundamental errors, regardless of the number of subjects involved, (2) responses can vary according to the subjects' interest in the experiment, and (3) the processes of adaptation to widely different stimuli, such as taking drugs, exposure to wave motion, or mere participation in an experiment, display certain common characteristics.

It may be queried whether the present findings could be peculiar to Asian races or tropical climates. Racial peculiarities can be excluded, however, since the students belonged to several races, while the Service men and women were all British. A psychological study (Carpenter, 1949), moreover, provides no evidence that the climate of Singapore was conducive to such symptoms as drowsiness and fatigue, though occasional laziness and loss of memory were noted. Finally, dryness

of the mouth resulting from chronic dehydration is unlikely in the climate of Singapore, which is humid and not very hot.

Summary

1. The side-actions of *l*-hyoscine hydrobromide, promethazine hydrochloride, and diphenhydramine hydrochloride were studied in experiments designed to give quantitative data that could be objectively analysed.

2. The results suggest that the side-effects of these drugs are proportionate to their preventive action against motion sickness.

3. The results are compatible with the view that motion sickness remedies have a common hyoscine-like action.

4. Some tolerance to the side-effects of hyoscine and promethazine was acquired when these drugs were given repeatedly for four days.

5. Evidence was found that experimental procedures by themselves can give rise to responses in human experimental subjects and that habituation to diverse experimental procedures may follow a common pattern.

I wish to thank Mr. Yap Tian Beng and Mrs. R. M. Guest, who in turn helped to run these experiments, and Mr. J. Hanam, of the Department of Pharmaceutics, University of Malaya, who prepared the drugs and kept the code. I am grateful also to the members of the University of Malaya and the British Army who acted as experimental subjects.

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VASOPRESSOR, ANTIDIURETIC, AND OXYTOCIC ACTIVITIES OF EXTRACTS OF THE DOG'S HYPOTHALAMUS

BY

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Abel (1924) has shown that pressor and oxytocic activities are present in extracts of the hypothalamus of the dog after careful removal of the pituitary stalk and median eminence. He had no doubt that he was dealing with the same substance which he had described in extracts of the posterior lobe. Though similar results were obtained by Trendelenburg (1928) and Sato (1928), later workers came to doubt the accuracy of these findings (Rioch, 1938), till the reinvestigation of this problem by Bargmann and his school confirmed and extended the old observations (Bargmann, 1949; Hild and Zetler, 1951; Hild, 1951).

In the course of some work on the distribution of sympathin in the brain of the dog, acid alcoholic extracts of the hypothalamus were submitted to paper chromatography in phenol HCl, and eluates of the different regions of the chromatogram tested biologically. When eluting a strip adjacent to but not including the solvent front of the paper, a strong pressor activity of the eluate was noticed when the extracts had been prepared from hypothalamus, whereas this activity was lacking in extracts of other parts of the central nervous system. The position on the chromatogram excluded noradrenaline and adrenaline as substances responsible for the effect, and it was possible that it was due to a vasopressin-like substance. Abel (1924) had shown that posterior lobe hormones are soluble in 95% alcohol, so that the unorthodox method of extraction did not preclude this possibility. The paper deals with the properties of these hypothalamic extracts.

METHODS

Dogs and cats were anaesthetized with chloroform and bled to death.

Preparation of Extracts.—0.1–0.25 g. brain tissue, which had been stripped of its pia mater, was homogenized in a glass grinder containing 1 ml. chilled absolute ethanol acidified with HCl (1 ml. conc. HCl per l. ethanol). The homogenate was transferred to a centrifuge tube with more (3.5 ml.) acid ethanol, chilled and spun; the supernatant was poured into a wide tube, and the residue washed with 1 ml. acid ethanol and spun. The combined supernatants were evaporated *in vacuo* (bath temperature 50° C.), the residue detached from the tube and transferred into a microtube with the aid of 0.5 ml. H_2O and four portions of 0.25 ml. 0.001 N HCl. The aqueous suspension was chilled and centrifuged; the supernatant was poured off and evaporated *in vacuo*. The new residue was detached from the tube with 0.5 ml. ethanol-acetone mixture 1:1, centrifuged, and the supernatant applied to paper for chromatography. The residue was treated with a further 0.25 ml. of acetone-alcohol mixture and centrifuged, and this supernatant was also applied to the paper. The chromatography, elution, and preparation of extracts for biological assay is described in a previous paper (Vogt, 1952a). The purpose of this procedure is to separate sympathomimetic amines from other constituents of the brain tissue and is not intended for quantitative extraction of posterior lobe principles.

Assays.—Commercial posterior lobe extract (Parke, Davis) served as a standard. All eluates to be assayed were prepared from the top 5 cm. of the chromatograms (excluding the actual front).

Pressor activity was assayed on the blood pressure of the hexamethonium-treated rat as described by Outschoorn (1952).

Oxytocic activity was assayed on the rat's uterus. The organ was suspended at 30° C. in a modified Ringer's solution containing only 0.006% $CaCl_2$ (see Gaddum, Peart, and Vogt, 1949) in order to suppress the spontaneous activity.

Antidiuretic activity was assayed by intravenous injection either into the trained, conscious, hydrated dog, or into rats anaesthetized with 10% ethanol following the procedure of Ames and van Dyke (1952). Comparison was always made with posterior lobe extract injected intravenously during the course of the same experiment.

The *inactivation* of posterior lobe activity was carried out by adding N-sodium thioglycollate, freshly prepared from the acid with H_2O and $NaHCO_3$, in a proportion of 1:100 to the extracts, adjusting the *p*H to not less than 7.4 and allowing the mixture to stand at room temperature for 20–40 min.

The 5-hydroxytryptamine salt used was the creatinine sulphate, the strength being stated in terms of the base.

Results

Several observations were made which demonstrated that the pressor substance in hypothalamic extracts did, in fact, greatly resemble posterior lobe hormone.

1. The extracts showed the same biological activity when their pressor and when their antidiuretic potencies were assayed against commercial pituitrin.

2. Dog posterior lobe tissue was extracted, chromatographed, and eluted in exactly the same manner as hypothalamic tissue. The biological activity was recovered from the same strip of the chromatogram (next to the solvent front) which had contained the activity in hypothalamic extracts.

3. Incubation, at room temperature and for periods of about 30 minutes, of hypothalamic extracts with 0.01 M sodium thioglycollate at *p*H 7.4 completely destroyed their pressor, antidiuretic, and oxytocic activity. This test is considered by van Dyke, Chow, Greep, and Rothen (1942) to be specific for posterior lobe hormone.

There was, however, one point in which hypothalamic extracts differed from posterior lobe extracts, and that was their oxytocic activity. Table I shows a series of parallel assays of the different biological activities of hypothalamic extracts in terms of international units of posterior lobe powder. It will be seen that, though agreement between pressor and antidiuretic activity is satisfactory, the oxytocic activity is invariably far below the expected value, the ratio, pressor over oxytocic activity (" P/O "), having a mean of 14.6.

Table I

EXTRACTS OF HYPOTHALAMUS OF THE DOG

Activity in m.u./g. fresh tissue

Part of Hypothalamus Extracted	Pressor	Anti-diuretic	Oxytocic	P/O	Age
Whole	1,040		150	7	Adult
Ventral	4,800		120	40	Adult
	4,000	5,000	240	17	Adult
Ventral, no median eminence	2,400	2,400	200	12	Adult
	4,000		240	17	Adult
	800		80	10	10–11 weeks
	300*		30	10	10–11 weeks
	520		40	13	10–11 weeks
	320		16	20	10–11 weeks
	400*		24	17	10–11 weeks
				M = 14·6	

* Injected with morphine hydrochloride 5 and 2 hr. before bleeding.
M = Geometric mean.

The possibility was then investigated that the oxytocic assays gave too low values owing to inhibitory substances present in the eluates. Standard oxytocin was added to eluates in which the hypothalamic hormone had been inactivated by incubation with 0.01 M thioglycollate, and the mixture immediately assayed: no inhibition of the activity of the standard was observed. The conclusion was, then, that hypothalamic extracts contain a substance akin in its properties to posterior lobe hormone, except for a much weaker oxytocic activity.

There was one more point which required checking. Amin, Crawford, and Gaddum (1952, personal communication) found that 5-hydroxytryptamine is present in the hypothalamus. Since this substance stimulates the rat's uterus at low concentrations, and since it travels in the chromatogram to a region which is adjacent to that from which the posterior lobe-like activity is recovered, it was essential to rule out the possibility that the oxytocic activity found in the eluates was due to 5-hydroxytryptamine.

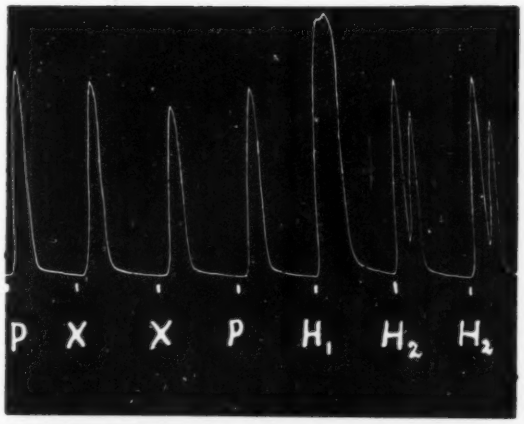
As also shown by Amin *et al.*, dihydroergotamine abolishes the oxytocic action of 5-hydroxytryptamine in doses which leave posterior lobe activity unaffected. A rat's uterus was suspended in a 2 ml. bath and contractions of comparable height were elicited by 0.35 m.u. pituitrin (" P "), 0.005 ml. hypothalamic extract (" X "), and 12.5 ~~μ~~ng. 5-hydroxytryptamine (" H_2 "). The contractions are seen in the top part of Fig. 1. Then 10 μg. dihydroergotamine methane sulphonate was added to the bath, left in for 11 minutes, washed out, and H_2, P, and X again added to the bath. The lower part of Fig. 1 shows that the effect of 5-hydroxytryptamine had been suppressed by the dihydroergotamine, whereas the effects of pituitrin and hypothalamic extract were precisely the same as before.

There is thus no reason to doubt that what oxytocic activity is exerted by the hypothalamic extracts is due to a substance which has biological and chemical properties similar to those of posterior lobe hormone.

In order to be certain that the chemical procedure adopted was in no way responsible for the low oxytocic activities obtained, extracts were made of the posterior pituitary of three dogs, treated by the same procedure as hypothalamic extracts and assayed for pressor, antidiuretic, and oxytocic activity. Table II shows the agreement between the different assays and an average pressor/oxytocic ratio of 1.13 which does not significantly differ from 1. Since the acid alcohol

ERRATA (June, 1953)

p. 194, *right-hand column, 3rd para., line* 7,
For 12.5 μg. *read* 12.5 ng.



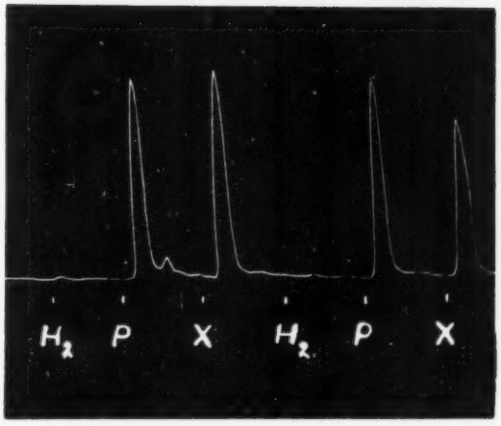


FIG. 1.—Uterus of oestrous rat, suspended in 2 ml. bath, stimulated by drugs every 4 minutes. P=0.35 m.u. pituitrin P.D. X= 0.005 ml. hypothalamic extract, corresponding to 0.0012 g. fresh tissue. The extract was administered as a 1 : 5 dilution in 0.9% NaCl. $H_1 = 50$ µg., $H_2 = 12.5$ µg. 5-hydroxytryptamine. Between the upper and the lower tracing interval of 15 minutes, during which 10 µg. dihydroergotamine methane sulphonate was added to the bath and washed out 11 minutes later.

TABLE II
EXTRACTS OF POSTERIOR LOBE OF THE DOG
Activity in m.u./g. fresh tissue

Dog No.	Pressor	Antidiuretic	Oxytocic
1	74,000		120,000
2	160,000		80,000
3	120,000	80,000	104,000

used in these experiments cannot be relied on to extract the whole activity, the figures in Table I may be underestimates, but they show, seeing that the weight of tissue employed ranged from 150 to 200 mg., that the amount of vasopressin in the adult brain is not less than about 1 i.u. Most of this activity was present in the ventral hypothalamus, from which the median eminence had been carefully removed in order to prevent contamination with pituitary tissue. The hormone was also found in the cat's hypothalamus. No activity (<2.5 m.u./ml.) was detected in dog's cerebrospinal fluid. Table I (last 5 figures) also indicates that young dogs have a lower hypothalamic hormone concentration than adult ones. The lowest figures ever observed, however, were obtained in puppies injected with morphine; some of these figures are not entered in the table because the activities were too small for an accurate assessment of the oxytocic effect. The action of drugs is under investigation with methods which ensure quantitative extraction of the hormone.

p. 195, *legend to Fig.* 1,
For $H_1 = 50$ µg., $H_2 = 12.5$ µg. *read* $H_1 = 50$ n
$H_2 = 12.5$ ng.

DISCUSSION

It is apparent from this work that the posterior lobe-like activity found in the hypothalamus of the dog is due to a substance differing in its properties from the hormone stored in the pituitary gland. Hild and Zetler (1951) also report a ratio of vasopressor/oxytocic potency which is greater than 1, but, as their ratio for the pituitary showed the same trend, they came to the conclusion that this was a general property of dog's posterior lobe hormone, irrespective of its localization. Since the authors were unaware of the presence of rather high concentrations of noradrenaline (1–2 µg./g. fresh tissue, Vogt, 1952b) in the hypothalamus, their estimation of hypothalamic pressor activities may have been subject to some inaccuracy; and in the important work of Zetler (1952), demonstrating the depletion of hypothalamic posterior lobe-like activity by thirst, the error introduced into the pressor assays by sympathin might, indeed, not have been negligible, since any contamination would have a greater influence on the results when the vasopressin present was only a fraction of the normal. If this were the correct explanation of the relatively high values reported by Zetler for vasopressor activity, there would be no need to postulate a dissociation of vasopressor from antidiuretic activity in the hypothalamus. This would certainly bring Zetler's results more in line with the view that these two activities are due to the same substance.

Kovács and Bachrach (1951) report that in experiments still in progress carried out by Kovács, Oláh, and Varró hypothalamic extracts of dogs had a greater antidiuretic than oxytocic potency. In contrast to the findings in the dog, this ratio was found to be unity in human material (Hild and Zetler, 1952). If these species differences are confirmed, they introduce a further complication into the problem.

Bargmann (1949), and Bargmann and Scharrer (1951), have put forward the theory that the

"posterior lobe" hormone is synthesized in the supraoptic and paraventricular nuclei and travels down the supraoptico-hypophyseal tract into the pituitary where it is stored. If we accept this theory, it would appear from the present work that the hormone, as synthesized in the ganglion cells, undergoes some chemical modification within the posterior lobe which enhances its relative oxytocic activity. Van Dyke, Chow, Greep, and Rothen (1942) have shown that a homogeneous protein can be prepared from posterior lobe tissue which has the same ratio of vasopressor to oxytocic activity as posterior lobe extracts. If the corresponding activities of hypothalamic extracts were also attached to a single molecule, it is this molecule which should undergo modification on its journey into the posterior lobe.

Recent experiments by du Vigneaud (1952) and Popenoe, however, suggest another interpretation. These authors prepared a highly purified vasopressin which did not contain any oxytocin, as shown by the complete absence from the preparation of leucine and isoleucine, two essential constituents of the oxytocin molecule. This vasopressin, when assayed on the guinea-pig's uterus, exhibited oxytocic activity of the order of 5% of the vasopressor potency. The authors therefore conclude that pure vasopressin has some intrinsic oxytocic activity. Comparison of the figure of approximately 5% for the intrinsic oxytocic activity of pure vasopressin and of that of 7% for hypothalamic hormone suggests that the substance extracted from the hypothalamus may be pure vasopressin.

It would be of great interest to know whether a secretion which is essentially vasopressor and very little oxytocic is ever given off into the blood stream. The only available evidence on the properties of the hormone circulating in the blood is that obtained by Harris (1947, 1948) in the rabbit. When the infundibular stem was stimulated electrically, and the effects on uterine activity and antidiuresis or vasoconstriction compared, the ratio vasopressor/oxytocic activity was not greater but smaller than 1 and amounted to 0.2–0.4. Unless we are here confronted with species differences, or with transformations of the hormone in the blood itself, Harris's results would therefore suggest that the circulating hormone was not identical either with the substance present in the hypothalamus or with that stored in the posterior lobe.

Summary

Extracts of the hypothalamus of the dog were assayed for pressor, antidiuretic, and oxytocic activity against pituitary (posterior lobe) extracts. The oxytocic activity was only about 7% of the pressor and antidiuretic activities.

Extracts of dog's posterior lobe, prepared in exactly the same way as the hypothalamic extracts, showed no such discrepancy between the various biological activities.

Before testing, all extracts were purified by paper chromatography so that the eluates used for biological assay were free of noradrenaline, adrenaline, and 5-hydroxytryptamine.

If one accepts the theory that the posterior lobe hormone is manufactured in the ganglion cells of the hypothalamus, these findings suggest that a modifying influence is exerted by the posterior lobe tissue on the molecule as it travels down to its site of storage in the pituitary. An alternative explanation would be that the hypothalamic nuclei synthesize vasopressin, whereas oxytocin is manufactured in the posterior lobe itself.

My thanks are due to Dr. Mary Pickford for allowing me to carry out antidiuretic assays on one of her trained dogs. It is a pleasure to thank Mrs. S. K. Pride for her excellent assistance.

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Brit. J. Pharmacol. (1953), **8**, 197.

PHYSICO-CHEMICAL PROPERTIES AND TOXICITIES OF DIFFERENT IRON PREPARATIONS*

BY

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After the preparation and standardization of saccharated iron oxide (Nissim and Robson, 1949a), a comparative study was made of this and other iron preparations with the object of gaining a clearer understanding of their pharmacological properties and of the manner in which iron compounds produce their toxic effects in the body. The iron preparations studied comprise both old and new compounds or complexes. The new forms were made during the search for other intravenous iron preparations among interaction products of iron with substances related to sucrose, including monosaccharides, disaccharides, caramelization products, and ascorbic acid. In addition, some amino-acids and protein hydrolysate were tried. Among the older preparations of iron a large number of readily available or easily prepared compounds were also investigated.

Methods and Materials

The *p*H at which precipitation took place in aqueous solution on the addition of HCl to alkaline iron preparations and on the addition of alkali to acid preparations was determined. The effect of the addition of plasma (5 ml. to an equal volume of 0.2 g. Fe per 100 ml. of solution of the iron preparation) on the precipitation point was studied as an indication of whether the preparation was likely to precipitate when injected intravenously or not. The *p*H at which a blue colour was obtained on the addition of potassium ferrocyanide to ferric preparations, or of potassium ferricyanide to ferrous preparations, was used to indicate the ease with which the iron compound was split *in vitro*.

The toxicity of the iron preparations was tested in female white mice. With the exception of saccharated iron oxide and ferric glucosate, the toxicity figures given here are only approximate, for the toxicity tests were concerned with wide differences significant enough for the purpose of the present study. In addition to the LD50—at one week from the injection—the immediately lethal dose, i.e., the dose which causes death while the preparation is still being injected (ILD), was also determined.

Ferric Glucosate.—The interaction of iron with glucose took place more readily than with sucrose (Nissim, 1949; Nissim and Robson, 1949b). Much less heat was required for the formation of this compound. The ferric hydroxide was prepared as described for saccharated iron oxide, and was mixed with glucose and sodium hydroxide (Analar). By analogy with ferric sucrate (Mackenzie, 1913), this preparation was termed ferric glucosate. The proportions of glucose to iron could be varied, but much smaller quantities of glucose were required than of sucrose, so that samples of ferric glucosate could be made with a glucose content ranging from 0.33 g. to over 33.3 g. per g. of elemental iron. The glucose content of the sample studied here was 1.8 g. per g. of iron. Alkali was added to the mixture of ferric hydroxide and glucose to bring the *p*H to 11.7, and the amount required was found to rise with the glucose content of the sample. With the application of moderate heat (e.g., 60° C.), the *p*H gradually fell. The *p*H at which precipitation of the sample took place on the addition of dilute HCl also fell from 8.0 to as low as 2.7 with continued heat. There was also a corresponding gradual fall in the *p*H at which the Prussian-blue reaction was given on the addition of potassium ferrocyanide and HCl from 7.0 before heating to 2.0 when the precipitation point had dropped to *p*H 2.7. In other words, the binding of iron in the preparation became firmer. As with saccharated iron oxide, the progressive fall in the *p*H at which precipitation occurred and in the *p*H at which the ferrocyanide test was given was accompanied by a reduction of toxicity.

Solutions of ferric glucosate could not be autoclaved, as precipitation always took place with the fall of *p*H below 10.5.

"Ferric Hydroxide Ferrous Ascorbate."—Ascorbic acid had such a remarkable affinity for iron that it dissolved ferric hydroxide (prepared from ferric chloride and sodium carbonate) completely without the aid of alkali or heat, giving rise to a purplish-black solution. The reaction took place more rapidly at 60–70° C. The two substances were used in the proportion of 20 g. ascorbic acid to 1 g. Fe. Sodium

* This work formed part of an M.D. thesis approved by the University of London.

hydroxide was added to the ascorbic acid/ferric hydroxide solution after 36 hours, and the *p*H brought up from 3.5 to 11.0 so that toxicity tests would be more comparable with those on saccharated iron oxide. "Ferric hydroxide ferrous ascorbate" is almost colourless at *p*H 1.5. It acquires a purplish colour when alkali is added, and the colour darkens until it becomes almost black at *p*H 11.0.

"*Ferrous Chloride Ascorbate.*"—This was prepared by combining 1 g. of iron as $FeCl_3$ with 20 g. ascorbic acid and bringing the *p*H up to 11.0. Immediate reaction occurred between the two substances at room temperature.

"*Ferric Chloride Caramelate.*"—According to the *B.P.C.* (1949), caramel is prepared by heating sucrose at 180–200° C. until a black viscid mass is formed. This is then mixed with sufficient hot water to produce a liquid of specific gravity 1.4. The degree of caramelization obtained with this method, and consequently the constituent products of the caramel, must vary a great deal from one preparation to another.

When sucrose was caramelized by heat both loss in weight and fall in *p*H were noted. These changes were used as a measure of the degree of caramelization, and repeated experiments with iron and standardized caramel samples have given consistent results. The samples of caramel used in these experiments showed a loss in weight of 6% and a fall in *p*H from about 5.7, which is the *p*H of a 4% (w/v) sucrose solution before caramelization, to 3.0.

When caramel was mixed with a solution of ferric chloride it gave a dark brown solution; a precipitate formed when sodium hydroxide was added, but dissolved again when a *p*H of about 11.4 was reached. When this solution was heated at 100° C. for one hour, the resulting preparation did not precipitate at any *p*H in aqueous solution. The proportion of iron to caramel in this preparation was 1:16.6. The *p*H of the solution dropped when heated to about 9.5 and was adjusted to 11.0 with sodium hydroxide.

Ferrous Pyruvate.—Pyruvic acid and ferric hydroxide were left overnight at 50–60° C.; 25 ml. of a 57% (w/v) solution of pyruvic acid dissolved 1 g. of iron as ferric hydroxide, reducing it to the ferrous state. This ferrous pyruvate, however, precipitated when alkali was added, at *p*H 7.0, and remained precipitated at all *p*Hs on the alkaline side. It was not considered worth further investigation.

"*Ferrous Chloride Pyruvate.*"—Pyruvic acid was mixed with ferric chloride in the same proportion of 25 ml. of 57% (w/v) pyruvic acid to 1 g. of iron and left overnight at 50–60° C. It formed a preparation which remained in solution when the *p*H was brought up from 0.9 to 11.0, but nevertheless proved more toxic than any of the preparations so far described.

"*Ferric Chloride Pyruvate.*"—Lactic acid reacted with ferric chloride instantaneously to give a lemon yellow solution which did not give a precipitate at any *p*H. Forty ml. of lactic acid was required to keep in solution in this form 1 g. of iron.

Protein Hydrolysate and Iron.—Protein hydrolysate (Casydrol-Bengers) gave a port-wine coloured solution with ferric chloride. On the addition of alkali a precipitate formed which redissolved with further alkali at a *p*H of about 8.0. The *p*H of the solution was brought up to 11.0 as with other preparations.

Plasma and Iron.—When plasma is mixed with ferric chloride its proteins precipitate. With the addition of alkali this precipitate turns into an almost solid jelly, but redissolves at a *p*H of 11.5–12. This solution may be neutralized with dilute acid without precipitating, its precipitation point being 6.8. The proportion of iron to plasma used was 0.4 g. to 100 ml.

Ferric Tartrate.—This is a scale preparation with an iron content of 19.46%.

Results

New Iron Preparations

Ferric Glucosate.—The *p*H values for the precipitation point and the ferrocyanide test as well as the *p*H at which the preparation precipitated when mixed with plasma (Table I) were all lower than those obtained with saccharated iron oxide, and on this account ferric glucosate was expected to be less

TABLE I

PHYSICO-CHEMICAL PROPERTIES AND TOXICITIES OF DIFFERENT IRON PREPARATIONS

Preparation	*p*H	*p*H of Precipitation Point	Plasma Precipitation Point	Ferrocyanide or Ferricyanide Test(*p*H)	LD50 mg. Fe/kg.	Immediately Lethal Dose mg. Fe/kg.
Saccharated iron oxide, sample A	10·7	5·7	7·2	3·5	180	450
Saccharated iron oxide, sample G_4	11·0	3·7	7·2	2·0	300	1,000
Ferric glucosate	11·0	2·7	6·8	2·0	150	600
Ferric hydroxide ferrous ascorbate	11·0	—	6·5	4·8	45	90
Ferrous chloride ascorbate ..	11·0	—	7·0	4·5	30	45
Ferric chloride caramelate ..	11·0	—	5·0	5·5	90	450
Ferrous chloride pyruvate ..	11·0	—	7·5	4·5	33	50
Ferric chloride lactate ..	11·0	—	—	6·5	16·5	45
Iron and protein hydrolysate	9·5	6·8	Not tested	Not tested	33	180
Ferric chloride and plasma	11·5	6·8	—	Not tested	80	180
Ferric chloride..	1·5	2·6–3·6	2·6–6·5	7·0	11	15
Ferrous sulphate	4·0	4·0–8·5	6·0–8·3	9·0	11	22·5
Colloidal $Fe(OH)_3$	8·0	2·0	8·0	2·0	25	30
Iron and ammonium citrate	7·3	2·0	7·0	4·8	16·5	360
Ferrous ascorbate (neutral) ..	7·0	—	5·5	8·0	33	90
Ferrous ascorbate (alkaline) ..	11·0	—	6·0	8·0	33	270
Ferric tartrate ..	7·3	—	7·0	6·5	16·5	90
Ferronascine ..	6·5	3·0	7·0	7·0	16·5	360

toxic. Its LD50, however, was about half that of sample G_4 of saccharated iron oxide (Nissim and Robson, 1949a), and histological sections showed abundant precipitation of the iron. Details of the post-mortem and histological findings of this and other iron preparations will be given in a later paper.

Other Sugars and Iron.—Ferric hydroxide, fructose, and sodium hydroxide gave a preparation which was the same as ferric glucosate in physico-chemical characteristics and in toxicity. The disaccharides maltose and lactose formed soluble preparations with iron possessing toxicities twice as great as that of saccharated iron oxide. These disaccharides are reducing sugars, and are acted upon by alkali to give caramelization products via the simpler monosaccharides glucose and fructose (Thorpe, 1938). They would thus be expected to have the same toxicities as ferric glucosate.

"Ferric Hydroxide Ferrous Ascorbate."—This preparation possessed the remarkable property of not precipitating at any *p*H in aqueous solution. When mixed with plasma it began to precipitate at *p*H 6.5 on the addition of HCl, and its precipitation was not complete till a *p*H of 3 was reached. It was, however, much more toxic than either saccharated iron oxide or ferric glucosate.

"Ferrous Chloride Ascorbate."—This preparation possessed different physico-chemical properties and was more toxic than "ferric hydroxide ferrous ascorbate."

"Ferric Caramelate."—The interaction product of ferric hydroxide and caramel was closely similar to ferric glucosate in all its physico-chemical characteristics and was not therefore tested for toxicity.

"Ferric Chloride Caramelate."—When mixed with plasma, solutions of "ferric chloride caramelate" did not precipitate until a *p*H of 5.0 was reached. At this *p*H the plasma proteins themselves begin to precipitate even when not mixed with iron. When injected into mice, however, "ferric chloride caramelate" proved more toxic that saccharated iron oxide as well as ferric glucosate, though it was better tolerated than "ferric hydroxide ferrous ascorbate."

Ferric Lactate.—This precipitated at a *p*H of about 5.0 on the addition of alkali, and was not studied further.

"Ferric Chloride Lactate."—This iron preparation possessed the remarkable feature, alone among all the iron preparations tested, of not precipitating at any *p*H when mixed with plasma. Even when some of the plasma proteins precipitated at a *p*H less than 5.0, the "ferric chloride lactate" itself remained in solution. It was, however, even more toxic than the pyruvate compound.

Glycine and Iron.—Glycine reacted with ferric chloride to give a yellow brown solution which did not precipitate at any *p*H. This iron complex caused immediate death in doses of 90 mg. Fe/kg., however, and was not studied further.

Plasma and Iron.—The iron binding globulin of the plasma, siderophilin, can hold about 300 μg. Fe per 100 ml., or 15 mg. for the whole of the plasma in man. It appears from the present experiments that plasma may hold iron in yet another way which reduces its toxicity a great deal. A solution of this iron-plasma complex containing 0.2 g. Fe per 100 ml. of solution was injected into mice and proved some eight times less toxic than ferric chloride.

Old Iron Preparations

Iron Preparations Precipitating in Neutral Aqueous Solution

These include compounds of both the ferric and ferrous variety. The *p*H of their solutions and their precipitation points are recorded in Table II. All gave solutions of acid reaction with *p*H ranging from 2.6 to 6.0. The individual investigation of these compounds was not carried further, as it was thought that, whatever other properties they possessed, such compounds would invariably produce toxic effects due to precipitation. One example was taken, however, of the ferric and of the ferrous group for a more detailed study, so that a more accurate picture might be obtained of the type and mechanism of the toxic actions of such compounds. The two prototypes chosen were ferric chloride and ferrous sulphate.

TABLE II

IRON PREPARATIONS PRECIPITATING IN NEUTRAL AQUEOUS SOLUTIONS

Preparation	*p*H of Solution	*p*H of Precipitation Point
(*a*) *Ferric group*		
Ferric chloride ..	1·0–1·5	2·6
,, citrate	2·6	2·7
,, acetate	4·4	4·5
,, oxalate ..	3·3	3·4
,, formate ..	3·9	4·0
,, hypophosphite	3·7	4·0
,, pyrophosphate	4·5	7·0
,, glycerophosphate	5·7	7·0
(*b*) *Ferrous group*		
Ferrous sulphate ..	4·0	5·0
,, lactate ..	5·3	5·5
,, oxalate ..	6·0	6·5

Ferric Chloride.—Readily ionized ferric compounds are noted for their precipitation of proteins, the iron itself precipitating in the process. The *p*H of 2 g. Fe per 100 ml. of solution of ferric chloride was 1–1.5. In aqueous solution it precipitated between *p*H 2.5 and 3.5. The precipitate of ferric hydroxide did not, of course, redissolve with the addition of more alkali as when the ferric chloride was mixed with plasma. The precipitation of ferric chloride in plasma also began at *p*H 2.5, but was not complete till *p*H 6.5.

Because of its rapid protein precipitating effect, ferric chloride proved the most toxic of all iron compounds, especially when injected in a relatively concentrated form. Even as little as 9 mg. Fe/kg. resulted in immediate death, when the dose was given in a concentration of 0.2 g. Fe per 100 ml. of solution. To cause immediate death with 0.02 g. Fe per 100 ml. of solution, however, some 15 mg. Fe/kg. had to be given. The LD50 of 11 mg. shown in the Table was that of 0.02 g. Fe per 100 ml. of solution.

Ferrous Sulphate.—In contrast to ferric ions, ferrous ions do not precipitate proteins. The *p*H of 2 g. Fe per 100 ml. of solution of ferrous sulphate was about 4.0. The precipitation of ferrous sulphate in aqueous solution began at a *p*H just exceeding 4.0, and was not complete till *p*H 8.5. This agreed with the greater solubility of ferrous as compared with ferric ions. When ferrous sulphate was mixed with plasma, precipitation occurred gradually between *p*H 6.0 and 8.5. The immediate toxicity was less than that of ferric chloride, for its immediately lethal dose was about 22 mg. Fe/kg. Its toxic effects in smaller doses manifested themselves gradually, however, so that its LD50 was about the same as that of ferric chloride.

Iron Preparations Remaining in Solution at a Neutral pH

Colloidal Ferric Hydroxide.—The sample used was "Colliron" (Evans Medical Supplies). Its ILD was very close to its LD50 (Table I). Mice receiving 22.5 mg. Fe/kg. showed acute dyspnoea and convulsions immediately on injection. Those which survived this stage tended to recover completely. This was in contrast to the gradually developing toxic effects seen after administration of saccharated iron oxide, ferric glucosate, or iron and ammonium citrate; the acute onset of the toxic effects must have been caused by the sudden flocculation of colloidal ferric hydroxide in the blood.

Iron and Ammonium Citrate.—Iron and ammonium citrate (*B.P.*) contains 20.5–22.5% iron. It differed from other iron preparations so far tested in showing an ILD which was some 20 times greater than its LD50.

Ferrous Ascorbate.—A sample of ferrous ascorbate prepared according to the method of Maurer and Schiedt (1936) was divided into two portions; one was kept neutral and the *p*H of the other was brought up to 11.0 to render possible a strict comparison with "ferric hydroxide ferrous ascorbate." Both the neutral and the alkaline ferrous ascorbate proved more toxic than "ferric hydroxide ferrous ascorbate," and though both showed the same LD50 their ILD differed markedly.

Ferronascine (*Roche*).—This is the sodium salt of di(α:γ-dihydroxy-β:β-dimethyl-butyrate)-ferric acid. Solutions had a *p*H of 6.5, and did not precipitate at any *p*H, but with plasma it began to precipitate at a *p*H of about 7.0. On standing overnight at this *p*H, it converted the plasma to a soft gel. This effect was not observed with any of the other iron compounds.

It is seen from the account given here that iron preparations differ widely in their toxicity, their LD50s ranging from 11 to 300 mg. Fe/kg. The gap between the LD50s of the older iron preparations and the new high level of 300 mg. for saccharated iron oxide has been bridged by the other new iron preparations which have been recently investigated, e.g., "ferric hydroxide ferrous ascorbate," "ferric chloride caramelate," and ferric glucosate.

Summary

The physico-chemical properties of a number of iron preparations have been investigated, and their toxicities compared by tests on mice. Iron compounds exist with almost evenly spaced LD50s ranging from 11 to 300 mg. Fe/kg.

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INVESTIGATIONS ON THE HYPOTENSIVE EFFECT OF THE HYDROGENATED ERGOT ALKALOIDS

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The effects of the hydrogenated alkaloids of ergot on the circulation are extremely complex. When administered intravenously to the anaesthetized intact cat, these agents produce a fall in blood pressure (Rothlin, 1946). This effect is mainly due to a central action. After section of the spinal cord at the level of the first cervical vertebra (C1) and destruction of the medulla oblongata (i.e., in the spinal cat) these alkaloids produce an increase in blood pressure (Rothlin, 1946). The rise is brought about by a direct constriction of those vessels which, having been deprived of tonic innervation from the vasomotor centre, are especially sensitive to vasoconstrictor stimuli. In isolated vessels, e.g., cattle artery strips *in vitro*, blood vessels of the rabbit ear or of frog extremities, the administration of the hydrogenated alkaloids of ergot causes vasoconstriction, although not regularly (Rothlin, 1946). However, if the sympathetic pathways from the vasomotor centre to the vessels are intact, the intra-arterial injection of these alkaloids to anaesthetized cats, and to unanaesthetized human beings, leads to vasodilatation and not to vasoconstriction (Bircher and Cerletti, 1949 ; Barcroft, Konzett, and Swan, 1951). This peripheral vasodilator effect is presumably associated with a blockade of adrenergic impulses, since under suitable conditions a fall in blood pressure can also be produced in the spinal cat ; i.e., if sympathetic tone is restored by a constant intravenous infusion of adrenaline or noradrenaline and a long-lasting increase in blood pressure produced, the hydrogenated alkaloids of ergot cause a fall of blood pressure (Rothlin, 1949).

Therefore the fall in blood pressure produced in the anaesthetized intact cat by the hydrogenated alkaloids of ergot is not exclusively due to a central action. A peripheral blockade of adrenergic impulses may also play a role.

After transection of the spinal cord above the sixth thoracic segment, the administration of dihydroergocornine causes a rise of blood pressure, whereas after transection below this level it causes a fall (Bluntschli, 1948). It therefore seems necessary to have an uninterrupted pathway from the medulla oblongata down to a certain spinal segment in order that the central effects of this alkaloid may be transmitted to the periphery.

The purpose of the present investigation was to locate more precisely the central site of hypotensive action of the hydrogenated ergot alkaloids, as well as to acquire further information on the nervous pathways conducting this effect to the periphery.

We therefore studied the effects of hydrogenated ergot alkaloids in the cat after transection of the spinal cord at the level of the first and seventh thoracic vertebra (T1 and T7), to supplement previous findings. Their effects were also investigated in the decerebrated cat, in which the higher centres of autonomic regulation in the diencephalon are absent but the medulla oblongata is intact. In some experiments transection of the spinal cord at the level of the sixth cervical vertebra (C6), the first (T1) and the seventh thoracic vertebra (T7) was carried out after decerebration.

Since the central effects of these alkaloids must be transmitted to the periphery by autonomic efferent fibres, it was of interest to observe how their actions were affected by the interruption of autonomic efferent pathways. For this purpose the autonomic synapses, in the anaesthetized intact cat and in the decerebrated cat, were blocked by the injection or infusion of tetraethylammonium. Similar experiments were carried out after transection of the spinal cord at the level of T1 and T7.

Finally, the effects of the hydrogenated ergot alkaloids were studied in the spinal cat, after the spinal cord had been cut at the level of T7. In some experiments the spinal cord was completely destroyed.

METHODS

The experiments were carried out on 115 cats weighing 2.1–4.1 kg. In the intact animal anaesthesia was produced by subcutaneous administration of a mixture of chloralose (0.05 g./kg.) and urethane (0.50

g./kg.). In experiments on the decerebrated cat or the spinal animal, ether anaesthesia was used only during the operation. Decerebration was carried out just above the posterior quadrigeminal bodies after ligation of both common carotid arteries. In some experiments the vagi were cut.

Blood pressure was registered by a mercury manometer connected to the left carotid artery. Records of the renal and intestinal volumes were taken to obtain information on the blood flow through these organs. Renal volume (left kidney) was recorded plethysmographically by means of Roy's oncometer. Intestinal volume was recorded by means of a glass oncometer.

Dihydroergocornine methanesulphonate or a mixture of the methanesulphonates of the three alkaloids dihydroergocornine, dihydroergocristine, and dihydroergokryptine in equal proportions (Hydergine), or dihydroergotamine methanesulphonate were injected into the jugular vein in doses of 0.03 mg./kg. and 0.1 mg./kg.

For purposes of comparison, ergotamine tartrate was injected in doses of 0.03 mg./kg. and 0.1 mg./kg. intravenously in 21 experiments.

Tetraethylammonium chloride was injected (two injections of 10 mg./kg. at an interval of 4–10 minutes) or infused for 20–30 minutes in a concentration of 1:1,000 (1–2 mg./min.) to provide ganglionic blockade.

In most experiments adrenaline and noradrenaline were injected intravenously before and after the administration of the ergot alkaloids. The inhibition of adrenaline and noradrenaline by the hydrogenated ergot alkaloids will not be described in the present paper.

Results

In the Anaesthetized Intact Cat

Transection of the Spinal Cord at T7.—Section of the spinal cord at T7 in the cat under chloralose urethane anaesthesia caused a fall of blood pressure. The average blood pressure in eight cats at the beginning of the experiment was 171 (±12) mm. Hg.* In 12 animals after section at T7 the average blood pressure was 125 (±15) mm. Hg. Thus transection of the spinal cord reduced the blood pressure by approximately 27%.

Although the blood pressure was reduced after transection of the cord at T7, dihydroergocornine or Hydergine (0.03 mg./kg.) caused a further fall of 26 (±2) mm. Hg (average) in blood pressure in all six experiments (see Fig. 1).

Renal volume decreased because of the fall in blood pressure. There was generally little change in the volume of the intestines, but in isolated experiments it increased.

Thus the effect of the hydrogenated ergot alkaloids in the anaesthetized cat after section at T7 was fundamentally the same as that in the anaesthetized intact animal (Rothlin, 1946).

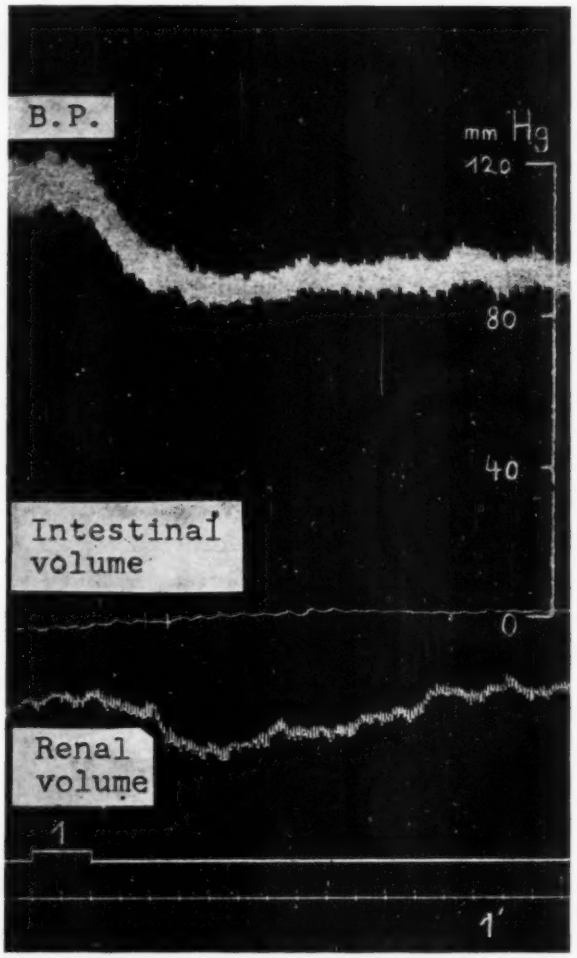

FIG. 1.—Hydergine reduces blood pressure in the cat after transection of the spinal cord at T7. Tracings from top to bottom are: blood pressure (mercury manometer connected to carotid artery), volume of the intestine and volume of the left kidney (plethysmography). Up-stroke=increase in volume, down-stroke=decrease in volume. Time in minutes. At 1, injection of 0.03 mg./kg. Hydergine.

Transection of the Spinal Cord at T1.—The average blood pressure in 26 animals after transection of the cord at T1 was 108 (±26) mm. Hg, i.e., approximately 63% of that in the intact animal and approximately 87% of that in the cat after transection at T7.

Doses of 0.03 and 0.1 mg./kg. dihydroergocornine and Hydergine had no uniform effect on blood pressure. In 10 experiments there was a fall (see Fig. 2*a*), in 7 there was a rise (see Fig. 2*b*), and in 5 there was no significant change.

There was no clear-cut relationship between the blood pressure values immediately before administration of the alkaloids and their effect on blood pressure.

* The figures in parentheses indicate standard deviation.

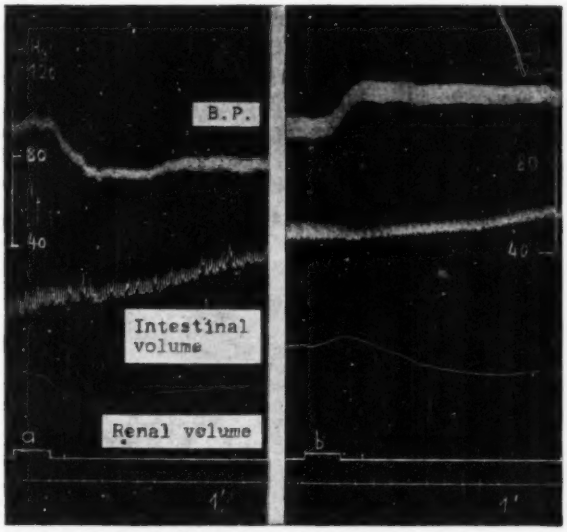

FIG. 2.—Effect of Hydergine and dihydroergocornine on blood pressure in two cats after transection of the spinal cord at T1. Tracings: from top to bottom as in Fig. 1. (*a*) Injection of 0.03 mg./kg. Hydergine. (*b*) Injection of 0.03 mg./kg. dihydroergocornine. See text.

Changes in renal volume were generally due to changes in blood pressure. There was very little change in the volume of the intestines.

In the Decerebrated Cat

The average blood pressure in 38 decerebrated cats was 126 (±17) mm. Hg. In 16 out of 17 experiments doses of 0.03 and 0.1 mg./kg. dihydroergocornine, Hydergine, and dihydroergotamine reduced the blood pressure (see Fig. 3). The average fall in blood pressure was 37 (±18) mm. Hg. In one experiment there was an increase in pressure.

The vascular reaction was principally the same as in the intact animal, i.e., the changes in renal volume were due to changes in blood pressure (see Fig. 3), and intestinal volume was only slightly affected.

Ergotamine, in contrast to the hydrogenated alkaloids, caused a rise of blood pressure in the decerebrated cat as in the anaesthetized intact animal.

Transection of the Spinal Cord at T7.—Transection of the spinal cord at T7 led to a decrease in blood pressure in the decerebrated cat as in the anaesthetized intact animal. The average blood pressure in 19 animals after transection of the cord was 96 (±21) mm. Hg, i.e., approximately 74% of that recorded before the operation.

The effects of 0.03 and 0.1 mg./kg. dihydroergocornine, Hydergine, and dihydroergotamine were studied in 14 experiments. In 11 cats the blood pressure fell by 22 (±14) mm. Hg (average), in one cat it rose, and in two cats there was no significant change. In the only experiment in which Hydergine (0.1 mg./kg.) produced a rise in pressure the initial blood pressure values were particularly low (66 mm. Hg). The vascular reaction

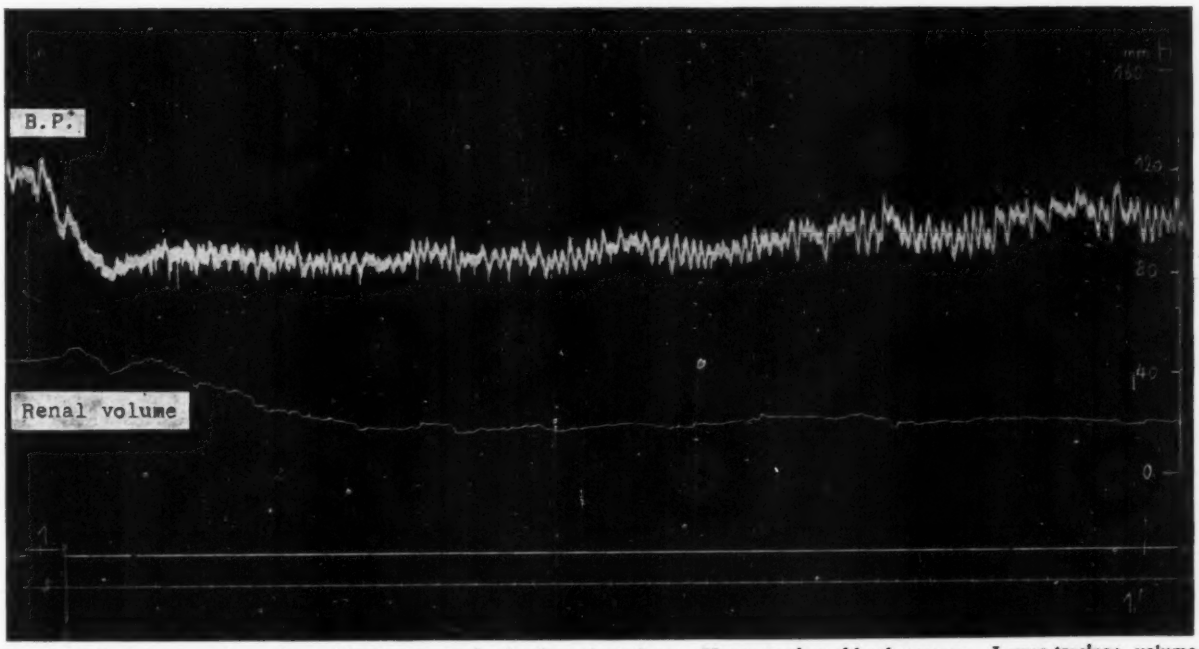

FIG. 3.—Dihydroergocornine reduces blood pressure in the decerebrated cat. Upper tracing: blood pressure. Lower tracing: volume of left kidney. Time in minutes. At 1, injection of 0.03 mg./kg. dihydroergocornine.

was identical with that in the decerebrated animal *not* subjected to transection of the spinal cord.

Ergotamine regularly increased blood pressure in the decerebrated cat both before and after transection of the spinal cord at T7.

Transection of the Spinal Cord at T1 and C6.—After decerebration and transection of the spinal cord at T1, the average blood pressure in 13 animals was 88 (±15) mm. Hg, i.e., approximately 70% of that after decerebration alone.

Doses of 0.03 and 0.1 mg./kg. dihydroergocornine, Hydergine, and dihydroergotamine given to nine animals had no uniform effect on the blood pressure. In three cats there was a decrease, and in six cats an increase. Changes in renal volume were due to changes in blood pressure.

Furthermore, after transection of the cord at C6 the hydrogenated ergot alkaloids exerted no uniform effect. In three experiments the blood pressure fell, in two experiments it rose, in one experiment it remained unchanged.

Ergotamine regularly produced an increase in blood pressure in the decerebrated cat after transection of the cord at T1.

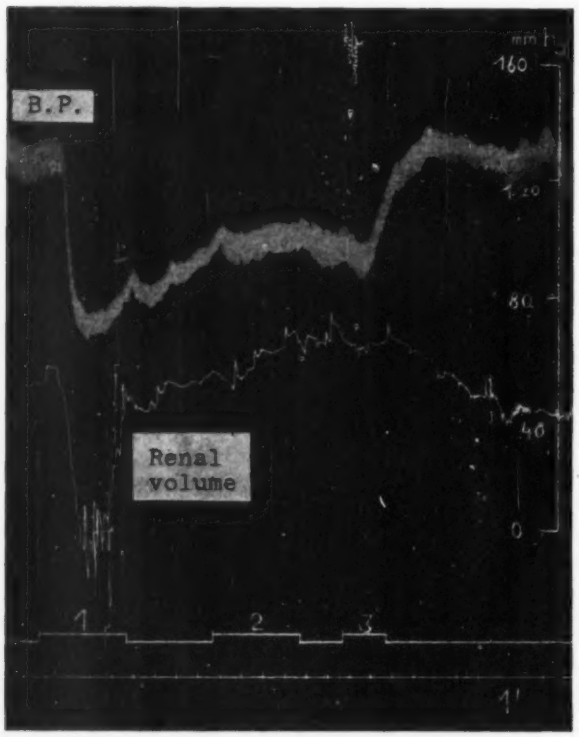

FIG. 4.—Dihydroergocornine increases blood pressure in the cat pre-treated with tetraethylammonium. Tracings from top to bottom as in Fig. 3. (1) Injection of 10 mg./kg. tetraethylammonium. (2) Injection of 10 mg./kg. tetraethylammonium. (3) Injection of 0.1 mg./kg. dihydroergocornine.

The Effect of Tetraethylammonium Chloride

The dose of tetraethylammonium employed by us (see Methods) blocks the majority of the autonomic synapses and thus prevents the transmission of stimuli from the centre to the periphery (Acheson and Moe, 1946; Moe and Freyburger, 1950).

The injection or infusion of tetraethylammonium produced a considerable fall in blood pressure in the anaesthetized intact and in the decerebrated animal. The injection of 0.03–0.1 mg./kg. dihydroergocornine, Hydergine, or dihydroergotamine after the injection or during the infusion of tetraethylammonium, i.e., when the blood pressure had reached a new level between 80 and 100 mm. Hg, generally caused a rise of blood pressure. In the intact animal the blood pressure rose in seven experiments (see Fig. 4) and fell in only one. After transection of the cord at T7 and administration of tetraethylammonium, the hydrogenated ergot alkaloids caused a rise of blood pressure (five experiments). Similarly, after transection of the cord at T1 and tetraethylammonium, the administration of these alkaloids led to an increase in blood pressure (four experiments).

In the decerebrated animal the blood pressure increased in six experiments and decreased in two.

In most of the animals treated with tetraethylammonium renal and intestinal volumes increased because of the rise in blood pressure. In some experiments they were reduced or showed no significant change.

The Spinal Cat after Transection or Destruction of the Spinal Cord

In the spinal animal the hydrogenated alkaloids of ergot always caused a rise of blood pressure after transection of the spinal cord at T7 or after complete destruction of the cord (see Fig. 5). Renal volume increased due to the rise in blood pressure.

DISCUSSION

The observations made show that the hydrogenated alkaloids of ergot, dihydroergocornine, Hydergine, and dihydroergotamine cause a fall of blood pressure provided that the medulla oblongata is intact and the spinal cord is intact as far down as the seventh thoracic segment (see Table I). The presence or absence of the diencephalon and cerebrum is not of importance, since a fall in blood pressure occurs in the decerebrated cat as well as in the anaesthetized intact animal. The primary importance of the medulla oblongata for the production of a fall of blood pressure is clearly shown in the experiments on the decerebrated cat. Thus, the diencephalon, in which Hess (1947) located

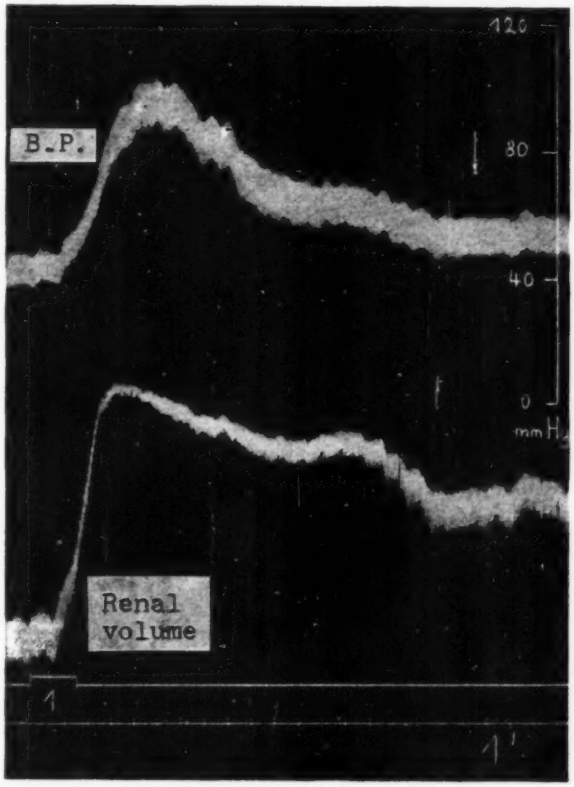

FIG. 5.—Hydergine increases blood pressure in the spinal cat after destruction of the cord. Tracings from top to bottom as in Fig. 3. At 1, injection of 0.03 mg./kg. Hydergine.

areas responsible for a syndrome accompanied by a fall in blood pressure, seems to play no significant role in the production of the hypotensive effect of the hydrogenated ergot alkaloids.

After the connection between the medulla oblongata and the spinal cord is broken at the level of the sixth cervical or the first thoracic vertebra, the hydrogenated alkaloids of ergot do not regularly produce a fall in blood pressure ; there may be an increase (see Table I) or no significant change. Thus our observations are not in complete accord with those of Bluntschli (1948), who reported that, after transection of the spinal cord at the level of the first thoracic segment, dihydroergocornine regularly caused an increase in blood pressure. The fact that we carried out a greater number of experiments may account for the differences between Bluntschli's findings and ours.

In any case, the effects of the hydrogenated alkaloids of ergot in the decerebrated cat after transection of the cord at T1 are similar to those in the anaesthetized intact animal after transection of the cord at the same level. In some experiments the blood pressure fell, in others it rose (see Table I).

It seems that when the spinal cord is transected at C6 or T1 some of the neural pathways which transmit the central hypotensive impulses to the periphery are interrupted and put out of action before they leave the spinal cord. If this is so, the direct vasoconstrictor effect of the hydrogenated ergot alkaloids may become predominant in some experiments and cause the blood pressure to rise.

Taylor and Page (1951) report that adrenaline and noradrenaline behave similarly. The injection of adrenaline or noradrenaline into the isolated perfused brain produces a fall in blood pressure throughout the general circulation if the spinal cord is intact but not if it has been cut at C6.

The direct vascular effect of the hydrogenated alkaloids of ergot is particularly impressive in the spinal cat (Rothlin, 1946). An increase in blood pressure is always observed in the spinal cat whether the spinal cord is intact, has been transected at T1, or completely removed (see Table I).

Which neural pathways remain for the transmission of central hypotensive impulses after transection of the cord at the level of the sixth cervical or the first thoracic vertebra? A bradycardia can be transmitted via the vagus, causing cardiac output to be reduced and blood pressure to fall (Rothlin, 1946). However, after bilateral

TABLE I

THE EFFECT ON BLOOD PRESSURE OF DIHYDROERGOCORNINE, HYDERGINE, AND DIHYDROERGOTAMINE IN DOSES OF 0.03 MG./KG. AND 0.1 MG./KG.

The figures indicate the number of experiments

Test Object	Blood Pressure		
	Fall	Rise	Unchanged
Anaesthetized intact cat after section of spinal cord at T1 ..	10	7	5
Anaesthetized intact cat after section of spinal cord at T7 ..	6	—	—
Decerebrated cat	16	1	—
Decerebrated cat after section of spinal cord at C6	3	2	1
Decerebrated cat after section of spinal cord at T1	3	6	—
Decerebrated cat after section of spinal cord at T7	11	1	2
Anaesthetized intact cat after pretreatment with tetraethylammonium	1	7	—
Anaesthetized intact cat after pretreatment with tetraethylammonium and section of spinal cord at T1	—	4	—
Anaesthetized intact cat after pretreatment with tetraethylammonium and section of spinal cord at T7	—	5	—
Decerebrated cat after pre-treatment with tetraethylammonium	2	6	—
Spinal cat after section of cord at T7	—	5	—
Spinal cat after destruction of cord	—	9	—

section of the vagi the hydrogenated alkaloids of ergot may still cause a fall of blood pressure. It is possible that the parasympathetic vasodilator nerves which originate in the medulla oblongata or the pons, such as are to be found in the chorda tympani, the nervus petrosus minor, and the nervus lingualis, play a role. Stimulation of these nerves liberates acetylcholine and may cause dilatation of the vessels of the salivary glands and tongue, as well as a fall in blood pressure (Feldberg, 1933).

The fact that the hydrogenated alkaloids of ergot caused a fall of blood pressure in only half of the experiments after transection of the cord at T1 in the intact cat as well as in the decerebrated animal, but in all experiments after transection of the cord at T7, suggests that there are neural pathways which leave the spinal cord between T1 and T7 and transmit the efferent hypotensive impulses to the organs in the periphery. The nerves concerned are most probably the sympathetic nerve fibres which transmit both vasoconstrictor and vasodilator impulses from the vasomotor centres to the periphery (Folkow and Uvnäs, 1948).

According to prevailing anatomical concepts no impulses from higher centres can be transmitted via sympathetic nerves to the peripheral organs after transection of the cord at C6 or T1, because the highest sympathetic preganglionic fibres in the cat leave the spinal cord at T1 or T2 (Fulton, 1943). However, after section of the cord at T7 the sympathetic preganglionic fibres which emerge above T7 can reach their effector organs in the head, the neck, the arms, the thorax (including the heart and the lungs), and certain portions of the abdomen. If the central effects of the hydrogenated ergot alkaloids are transmitted to the above-mentioned effector organs via the sympathetic nerves, the blood pressure falls. This does not exclude the probability that the organs supplied with sympathetic nerves from below T7 are also involved in the fall of blood pressure caused by these substances in the anaesthetized intact cat.

It has not yet been conclusively established whether the hydrogenated alkaloids of ergot depress the outflow of vasoconstrictor impulses from the vasomotor centre or whether they enhance the outflow of vasodilator impulses from the vasodilator centre, or whether both these mechanisms are involved.

In view of the fact that the hydrogenated alkaloids of ergot can exert a central inhibition of baroceptive reflexes (Rothlin, 1946; Sutton, Cerletti, and Taeschler, 1950), prevent central fluctuations in blood pressure (Rothlin and Cerletti, 1949), and potentiate the effects of analgesics (David and Semler, 1952), it is not improbable that they inhibit vasoconstrictor impulses centrally.

Further evidence that the fall of blood pressure caused by the hydrogenated alkaloids is of central origin and is transmitted to the periphery by autonomic pathways is provided by the observation that after tetraethylammonium the hydrogenated alkaloids of ergot cause a rise of blood pressure. Whereas tetraethylammonium intensifies the effects on blood pressure of substances with a peripheral site of action, such as histamine and mecholyl (Page and Taylor, 1950), it reverses the effects of the hydrogenated alkaloids of ergot, so that blood pressure rises instead of falling. This difference between the hydrogenated ergot alkaloids and vasodilator substances with a peripheral site of action is strong evidence that the fall of blood pressure caused by these alkaloids is predominantly of central origin. Their effect on blood pressure in the anaesthetized intact or decerebrated cat after chemical blockade of the autonomic efferent pathways is similar to that in the spinal animal. In the spinal animal the alkaloids increase the blood pressure because the destruction of the medulla oblongata prevents a central hypotensive action, and, consequently, the direct vasoconstrictor action predominates. After chemical blockade of the peripheral autonomic synapses the hydrogenated ergot alkaloids cause a rise of blood pressure because the transmission of the central hypotensive impulses through the sympathetic nerves is interrupted in the synapses and, consequently, the normally latent direct vasoconstrictor action predominates.

Therefore, the medulla oblongata is the main central site of hypotensive action of the hydrogenated ergot alkaloids, and the upper sympathetic fibres (down to T7) transmit the central impulses to the periphery.

SUMMARY

1. The hydrogenated alkaloids of ergot, dihydroergocornine, Hydergine (a mixture of equal parts of dihydroergocornine, dihydroergokryptine, and dihydroergocristine), and dihydroergotamine caused a fall of blood pressure in the anaesthetized intact cat and in the decerebrated cat, mainly by an action on the vasomotor centres in the medulla oblongata.

2. After section of the spinal cord at the level of the seventh thoracic vertebra, these substances regularly caused a fall in blood pressure in the anaesthetized intact cat and in the decerebrated animal.

3. After section of the spinal cord at the level of the sixth cervical or the first thoracic vertebra, these alkaloids did not regularly produce a fall in blood pressure in the anaesthetized intact cat and the decerebrated animal; in approximately half the experiments an increase in blood pressure was observed.

4. After ganglionic blockade by means of tetraethylammonium the hydrogenated alkaloids of ergot generally produced an increase in blood pressure in the anaesthetized intact cat and in the decerebrated cat.

5. An increase in blood pressure was always produced by the alkaloids in the spinal cat, even after section of the cord at the level of the seventh thoracic vertebra or after complete destruction of the cord.

6. Therefore, *the vasomotor centres, as the main site of action,* and *the upper part of the sympathetic outflow, as pathways conducting the impulses,* have to be intact for the hydrogenated alkaloids of ergot to cause a fall of blood pressure. When the vasomotor centres or the autonomic efferent fibres are put out of action, the normally latent vasoconstrictor effect of these alkaloids may predominate and produce an increase in blood pressure.

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THE REACTIONS OF RABBITS TO POISONING BY *p*-NITROPHENYLDIETHYLPHOSPHATE (E600)

BY

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The compound *p*-nitrophenyldiethylphosphate (E600) is an active inhibitor of acetylcholine esterase, and under the conditions in which this reaction has been studied *in vitro* the inhibition of the cholinesterase of red blood cells is apparently irreversible (Aldridge, 1950). Hobbiger (1951) has shown that the inhibition of red cell cholinesterase by tetraethylpyrophosphate is slowly reversible if observations are continued for a long enough period. Comparable observations with E600 have not been made.

The experiments recorded in this paper suggest that some reversal of the inhibition of cholinesterase in the animal may take place very rapidly and that successive inhibition by repeated doses of E600 can also be reversed. But simultaneously other irreversible changes are also taking place.

METHODS

Male rabbits of mixed breeds weighing 1–2.8 kg. were used. A Palmer "Ideal" respiration pump was used to provide the artificial respiration.

In order to facilitate artificial respiration in the unanaesthetized animals tracheotomy was sometimes performed under ether and a glass cannula tied in place. The wound was sewn up and the animal allowed to recover fully (one hour) from the effects of the ether.

Observations were made on the respiratory movements, the carotid arterial blood pressure, and the response of the tibialis anticus and soleus muscles to indirect electrical stimulation of rabbits anaesthetized with urethane 1 g./kg. intravenously. Ether was given only during the preparation of the tendons of the leg muscles immediately after preliminary tracheotomy. Under urethane alone the sciatic nerve was exposed and tied, and silver-wire electrodes placed beneath it. The nerve was stimulated once every two seconds by a square wave impulse of supramaximal intensity and 0.4 m.sec. duration. A lever attached to the liver by a thread recorded movements of the diaphragm. At the end of the dissection a further 0.5 g./kg. of urethane was given; no further anaesthetic was required. All drugs were given by the ear vein and all the animals were given repeated doses of atropine. Stock solutions of E600 in absolute alcohol were diluted either in saline or atropine immediately before use. Acetylcholine was administered as a freshly prepared solution in saline.

RESULTS

Reactions of Unanaesthetized Rabbits to E600

A single dose of 0.1 mg./kg. E600 given intravenously produced effects within 1–2 min. characteristic of poisoning by drugs which resemble acetylcholine. Atropine was given to the majority of animals in order to prevent salivation, bronchial secretion, bronchial spasm, and cardiac slowing. Widespread muscular fasciculations appeared and the animal collapsed with frequent kicking movements of the limbs. Respiration ceased and was soon followed by circulatory collapse as judged by the condition of the ear vessels. Death took place within 10–15 min. of injecting E600. But if artificial respiration was administered before the circulation collapsed, spontaneous breathing started within 2–5 min., and as it improved in amplitude and frequency the convulsive limb movements gradually disappeared. Within 25 min. of administering the E600 the animal could sit up, but showed an obvious general weakness and persistent widespread fasciculations.

As many as 10 successive doses of E600 have been given to a single rabbit over a period of 2½ hours. Each was followed by a rapid collapse and generalized limb twitchings, but with the help of artificial respiration the animal was restored to consciousness.

The periods of unconsciousness did not appreciably increase with successive doses. Other changes did appear. The generalized fasciculations but not the convulsive limb twitchings were very much reduced after several doses of E600 and almost disappeared. The failure of respiration

after 2–3 doses of E600 took a new form. Instead of a sudden arrest with progressive return after artificial respiration, breathing gradually slowed and became more shallow. Natural breathing could be restored by a period of artificial respiration, but might fail again in the same way several times before recovery was complete. The need for longer and more frequent periods of artificial respiration in order to restore natural respiration increased with successive doses of E600.

Anaesthetized Rabbits

Within 1 min. of injecting 1 mg./kg. E600 muscular fasciculations became visible and soon spread to involve most of the musculature. The movements of the diaphragm became very irregular until they ceased altogether, except for some fasciculations. At this stage the costal muscles might begin to operate and a few heaving chest movements take place. These soon ceased, cyanosis rapidly became extreme, and the blood pressure began to rise. After a further 1–2 min. the blood pressure fell rapidly and the heart slowed and soon stopped. During this asphyxial period the muscular fasciculations became very intense and twitching of the limbs sometimes occurred. If artificial respiration were started as soon as the blood pressure began to rise, recovery was certain, but this was not always so if the start of artificial respiration was delayed until the fall in blood pressure had begun. Immediately on starting artificial respiration by forced ventilation there was a sharp fall in blood pressure, presumably the result of interfering with the venous return to the heart by raising the intrathoracic pressure, but the colour improved and within a few minutes normal respiratory movements started. At first they were small in extent, but soon increased, so that the earlier rhythm and excursions were restored. As soon as oxygenation was improved tremors and fasciculations diminished, but the latter persisted after the full restoration of natural breathing.

If a succession of doses of E600 was given at intervals of 30–60 min. the diaphragm became refractory and ceased to respond by fibrillations and failure. Of nine rabbits receiving repeated doses of E600 in this way, four became refractory after one dose, four after two doses, and one after three doses of E600. Successive doses of E600 then had no immediate effect at all upon the excursions of the diaphragm. However, over the ensuing 3–5 hours the respiration rate gradually became slower and the diaphragm excursions smaller. There was also a slow but continuous fall in blood pressure unaffected by further doses of atropine. This gradual failure of both the respiration and circulation finally became complete and the animals died. The course of the decline could be temporarily arrested by prolonged periods of artificial respiration, which temporarily improved the blood pressure and the rate and extent of the diaphragm excursions. Seven animals receiving four or more injections of E600, totalling from 4 to 13 mg./kg., survived for 170–310 min., but the length of survival was not proportionate to the amount of drug administered.

The response of the leg muscles to indirect stimulation varied somewhat from animal to animal. But in all preparations there was some diminution of the contraction after each dose of E600, and this response was seen even after the diaphragm had become refractory to the drug.

The other most obvious change was the disappearance during the later stages of the poisoning of all the fine muscular fasciculations.

If it is assumed that E600 kills the animals because of the ability to inhibit cholinesterase, it is necessary to postulate that during the short periods of artificial respiration some at least of this process is reversed. Evidence that some cholinesterase might be released from inhibition was provided by the response of the rabbit to acetylcholine. A dose of 100 μg. acetylcholine given intravenously to rabbits that had received atropine had no effect upon the respiratory movements or response of the leg muscles to indirect stimulation.

When this same dose of acetylcholine was given to a rabbit revived by artificial respiration from a "lethal dose" of E600, the response of the stimulated leg muscles and natural respiratory movements were abolished or reduced for varying periods. The severity of the reaction diminished as the interval between the time of injecting the E600 and the acetylcholine was increased. If given 20 min. after E600, 100 μg. acetylcholine would arrest respiration and abolish the response of the leg muscles. But 100 min. later the same dose of acetylcholine had no effect on respiration and only reduced the response of the leg muscle by 10–15%.

When the animal was given a series of injections of E600, the response to 100 μg. acetylcholine given at the same interval after each dose of E600 did not change. Even though the natural respiration became unaffected by the later doses of E600 it remained equally sensitive to acetylcholine given after the E600.

One difference only was noted in the response of the rabbits to acetylcholine given after successive doses of E600. After the first or second dose of E600, acetylcholine produced a transient

shower of muscular fasciculations in the whole musculature, but this did not happen when the acetylcholine was given after later doses of E600. By this time spontaneous muscular fasciculations had also ceased.

Discussion

De Candole, Douglas, and Spencer (1950) studied rabbits poisoned by tetraethylpyrophosphate, which has an action very similar to that of E600. They found that at the time when the animal died the diaphragm no longer responded to indirect stimulation by way of the phrenic nerve. Death appeared to be due to a failure of nerve conduction at the periphery. This is apparently a very temporary failure after E600, though it may suffice to kill the animal in the absence of artificial respiration. If the diaphragm is inspected during the phase of acute poisoning it is seen to be the seat of irregular fasciculations, which are also widespread throughout the voluntary musculature.

Soon after the start of artificial respiration, with improved oxygenation of the peripheral blood the general fasciculations are much reduced, though they persist to some extent. After a few minutes of artificial respiration spontaneous respiratory movements start. Gradually the spontaneous fasciculations in the body musculature die down and at this stage injections of E600 no longer affect the excursions of the diaphragm.

Lundholm (1949) studied the effects of DFP on the rabbit and stated that there were two types of respiratory failure, one due to a failure of nerve-muscle conduction at the periphery and a second later stage which he attributed to central effects. Repeated doses of E600 in both anaesthetized and unanaesthetized rabbits result in a gradual slowing of respiration and failure of the circulation, which are unaffected by injections of atropine and are presumably due to the action of E600 on the central nervous system.

It is necessary to consider the role played by the inhibition of cholinesterase in the production of these signs of poisoning. Judged by the response of the anaesthetized rabbit to injections of acetylcholine, an inhibition of cholinesterase takes place after each of a succession of doses of E600. This inhibition is to some extent reversed in the course of the next two hours. But in two hours the reversal is never enough to restore the animal's normal degree of insensitivity to acetylcholine. Successive " lethal " doses of E600 do not make the animals more sensitive to acetylcholine, which suggests that the inhibition after the first dose is maximal. Reversal of this sensitivity to acetylcholine takes place with equal readiness after each of a series of injections of E600. Although the movements of the diaphragm are unaffected by the later injections of E600, they are still arrested by acetylcholine, which, if given within 10 min. of a dose of E600, may produce respiratory arrest of sufficient duration to require artificial respiration to prevent death.

The generalized muscular fasciculations gradually disappear after successive doses of E600. If the appearance and subsequent disappearance of these fasciculations are related to the degree of inhibition of cholinesterase at the myoneural junction, it is clear that a reversal of this effect is certainly not taking place in the intervals between the successive doses of E600. If the interval between doses is extended to 1–2 hours the fasciculations do return, and if no further doses of E600 are given will continue uninterruptedly for a further hour or two. During the early stages of poisoning the occurrence of these fasciculations apparently interferes to such an extent with the response of the diaphragm to natural and artificial impulses that complete failure of respiration occurs. But if the animal is tided over this critical period by artificial respiration, the diaphragm recovers sufficiently to respond to regular natural stimuli. This condition is not brought on again by successive doses of E600. As the death of the animal can be attributed to asphyxia, it is not unreasonable to suppose that asphyxia itself may make the condition at the diaphragm so much worse for the conduction of natural impulses from the phrenic nerve. This supposition is supported by the observation that throughout the voluntary musculature the fasciculations are intensified by the asphyxia and are immediately relieved when artificial respiration redresses the condition. Paton and Zaimis (1951) noted a similar effect on cats injected with decamethonium. The " curarizing " effect of this drug was enhanced during periods of anoxia due to respiratory failure. Apparently under conditions of asphyxia myoneural conduction is less efficient than when the blood is fully oxygenated. In the rabbit poisoned with E600, after 1–3 doses the diaphragm apparently overcomes this handicap to the conduction of natural impulses, and with the disappearance of the fasciculations further inhibition of cholinesterase by successive doses of E600 does not upset conduction.

In the unanaesthetized rabbit successive doses of E600 produce periods of unconsciousness accompanied by convulsive limb movements. If the inhibition of cholinesterase is responsible for the

production of these effects, this inhibition, too, must be readily reversed during the period of artificial respiration after each dose of E600. The duration of the period of unconsciousness is not significantly increased after each of a number of successive doses of E600.

Thus in anaesthetized and unanaesthetized animals sensitivity to acetylcholine and the production of unconsciousness go through a similar reversible cycle after a number of successive doses of E600, and these effects might be attributable to periodic complete inhibition of the cholinesterase at the periphery and in the central nervous system followed by a partial reversal of this inhibition.

In both types of animals slow progressive changes also take place, associated with a gradual slowing of respiration and failure of the circulation. It seems probable that these must be the result of some secondary changes brought about by a persistence of a considerable degree of cholinesterase inhibition in the tissues.

Summary

1. Rabbits, anaesthetized or unanaesthetized, die from acute respiratory failure within 10–20 min. of receiving a lethal dose of the powerful anticholinesterase *p*-nitrophenyldiethylphosphate (E600). The failure is apparently peripheral and can be overcome by a short period of artificial respiration.

2. Revival by artificial respiration may be effected after a series of such injections in the unanaesthetized rabbit.

3. After 1–3 such injections the anaesthetized rabbit no longer responds to further doses of E600 and respiration does not stop.

4. Gradual failure of the circulation and respiration develops after a series of injections of E600.

5. Sensitivity to acetylcholine increases after each dose of E600, and diminishes but does not return to normal within a 2-hour period.

6. Muscular fasciculations are widespread after the early injections of E600, but disappear and do not reappear after further doses of E600.

7. While part of the effect of E600 on cholinesterase can be reversed in the living animal, the development and disappearance of the muscular fasciculations are not part of a reversible cycle.

8. It is suggested that some of these changes may be a secondary reaction to the inhibition of cholinesterase.

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SOME OBSERVATIONS ON THE CHOLINESTERASES OF THE CENTRAL NERVOUS SYSTEM AFTER THE ADMINISTRATION OF ORGANO-PHOSPHORUS COMPOUNDS

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Three cases of paralysis in workers exposed to a new organo-phosphorus compound (mipafox, bis-mono-isopropylamino fluorophosphine oxide) have been reported (Bidstrup and Hunter, 1952). The condition was said to resemble closely the toxic effects of tri-ortho-cresyl phosphate (TOCP). Paralysis in man due to TOCP poisoning has been known since 1899 (Lorot). The lesion can be produced in certain laboratory animals ; in the chicken and rabbit it has been examined histologically (Smith and Lillie, 1931) and shown to consist of demyelination of peripheral nerves and some tracts in the spinal cord.

The effect of a number of newer organophosphorus compounds on the chicken has recently been studied. Di-isopropyl fluorophosphonate (DFP) and mipafox were found to produce demyelination identical with that produced by TOCP (Barnes and Denz, 1953). Earl and Thompson (1952b) paralysed chickens by TOCP and found that there was a marked inhibition of the pseudo cholinesterase of the central nervous system persisting until the onset of paralysis 10 days after giving the TOCP. Ord and Thompson (1952) had previously investigated the distribution of brain and spinal cord cholinesterases in man, and concluded that the pseudo cholinesterase appears to be associated with white matter. Further, TOCP, DFP, and mipafox selectively inhibit pseudo cholinesterase rather than true cholinesterase (Earl and Thompson, 1952a ; Aldridge, 1953 ; Ord and Thompson, 1950). Observations on the plasma cholinesterase levels in the human cases of poisoning by mipafox showed that the pseudo cholinesterase remained at a low level for many weeks (Callaway, Davies, and Risley, 1952). These observations led Earl and Thompson to make the interesting suggestion that pseudo cholinesterase might play a part in myelin metabolism and that inhibition of this enzyme in nervous tissue might therefore bring about demyelination.

Of the many organo-phosphorus compounds tested on chickens, only a few have been shown to be capable of producing demyelination and paralysis. There is no obvious correlation in their chemical structure or general properties as anticholinesterase poisons which distinguishes those that do from those that do not produce demyelination. An examination was therefore made of the changes in activity of the true and pseudo cholinesterase of the chicken's central nervous system and peripheral nerve after administration of both compounds which produce demyelination and those which do not, to see whether they differed in their effect upon these enzymes as suggested by Earl and Thompson's hypothesis. Their behaviour as selective inhibitors *in vitro* against chicken cholinesterase has also been studied.

METHODS

Warburg buffer and manometric methods used were as described by Aldridge (1950) and Davison (1953). Final substrate concentrations were acetylcholine perchlorate (AcCh) 0.0138 M, butyrylcholine perchlorate (BuCh) 0.03 M, and acetyl-β-methylcholine bromide (MeCh) 0.03 M. AcCh and BuCh were prepared in this laboratory, MeCh was obtained from Messrs. Light & Co. Ltd., Colnbrook.

The inhibitors employed were di-isopropyl fluorophosphonate (DFP) obtained from Dr. Saunders, Cambridge ; tri-ortho-cresyl phosphate (TOCP, Messrs. Geigy Ltd., Manchester) ; bis-mono-isopropylamino fluorophosphine oxide (mipafox, isopestox) and pyrophosphoric acid tetra (mono-isopropyl amide) (isoompa) from Pest Control Ltd., Cambridge ; O.O di-isopropyl O.p-nitrophenyl phosphate (iso-E600) and tetra-isopropyl pyrophosphate (TIPP) from Albright & Wilson Ltd., Birmingham. .

Adult hens of about 2 kg. weight and male albino rats of about 200 g. were used. Hens were killed by

injection of sodium pentobarbitone (Abbott Laboratories Ltd.) into the wing vein and rats by coal gas. The tissues were dissected out, chicken cerebrum and cerebellum separated, washed free from blood in saline, blotted dry, weighed, and homogenized with buffer in an all-glass homogenizer (Potter–Elvehjem type). Sciatic nerve was minced finely with scissors before homogenizing, and the cholinesterase activity was then determined manometrically in micro flasks (capacity about 1 ml.). Corrections were made for non-enzymic hydrolysis of substrates.

Results

Determination of True and Pseudo Cholinesterase in the Chicken.—MeCh, which is usually regarded as a specific substrate for true cholinesterase (Mendel, Mundell, and Rudney, 1943), has been shown by Earl and Thompson (1952a) to be hydrolysed both by the true and pseudo cholinesterases of the chicken, therefore MeCh hydrolysis does not give an accurate measure of true cholinesterase activity except in tissue such as the cerebrum, which contains very little pseudo cholinesterase. True and pseudo cholinesterases have therefore been estimated by a method depending upon the selective inhibition of pseudo cholinesterase with mipafox (Davison, 1953). The activity remaining after incubation of homogenates of brain and spinal cord with 1×10^{-7} M mipafox for 30 min. at 37° C. has been taken as true cholinesterase. The difference between this and the original uninhibited activity is the activity of pseudo cholinesterase. At a concentration of 1×10^{-7} M mipafox all the pseudo cholinesterase (brain, spinal cord, and plasma) is inhibited, while the activity of the true cholinesterase remains virtually unaffected. Thus the activity of true and pseudo cholinesterase of different tissues against AcCh and BuCh was determined. It is interesting to note that under these conditions the residual activity of chicken brain will only hydrolyse one part of BuCh for every 100 of AcCh, but in the spinal cord and sciatic nerve it hydrolyses 7 parts of BuCh for every 100 of AcCh. In the brain, spinal cord, and sciatic nerve, pseudo cholinesterase was found to hydrolyse 110 parts of BuCh for every 100 of AcCh. For the cholinesterases of spinal cord and sciatic nerve, the ratio of AcCh to BuCh hydrolysis ranged from 14.3 for 100% true to 0.91 for 100% pseudo cholinesterase. When both true and pseudo cholinesterase were active, intermediate values were found for this ratio, and these could be calculated arithmetically and were plotted on a calibration curve. From the AcCh/BuCh ratio determined for any time, the relative contribution of true and pseudo cholinesterase can be determined and its composition expressed in terms of hydrolysis of either AcCh or BuCh.

Inhibition of Chicken Cholinesterases in vitro.—As a preliminary to experiments in the living animal the inhibitor concentrations producing 50% inhibition of true and pseudo cholinesterase have been determined. The inhibitor concentration producing 50% inhibition of true cholinesterase, after incubation for 30 minutes at 37° C., divided by that for pseudo cholinesterase gives an indication of the selectivity of the organophosphorus compound. The results are presented in Table I, and show that all the compounds tested inhibit pseudo cholinesterase preferentially, but the degree to which they do so varies over a wide range.

Table I

CONCENTRATIONS OF DRUGS WHICH CAUSE 50% INHIBITION OF CHOLINESTERASES *IN VITRO*

Chicken brain and plasma preparations were incubated with different concentrations of inhibitors for 30 min. at 37° C. Cholinesterase activity was determined with MeCh for the true cholinesterase of brain and BuCh for the pseudo cholinesterase of plasma

Inhibitor	Molar Concentration Required to Cause 50% Inhibition		Ratio of These Concentrations
	True	Pseudo	
Mipafox	3×10^{-5}	1×10^{-9}	30,000
DFP	$1{\cdot}6 \times 10^{-7}$	2×10^{-9}	80
TOCP	—	—	67*
Iso-ompa ..	$4{\cdot}1 \times 10^{-5}$	1×10^{-6}	41
Iso-E600 ..	$3{\cdot}2 \times 10^{-7}$	$6{\cdot}3 \times 10^{-8}$	5
TIPP	$2{\cdot}4 \times 10^{-7}$	$1{\cdot}3 \times 10^{-8}$	18

* Calculated from Earl and Thompson's (1952a) data.

Inhibition of Cholinesterases after Injection of the Inhibitors in vivo.—The activity of true and pseudo cholinesterase, determined as described above by AcCh and BuCh hydrolysis, in the various parts of the nervous system (cerebrum, cerebellum, cervical and lumbar spinal cord, and sciatic nerve) of the normal chicken was measured in 6 birds. The results are given in Table II. Similar measurements were also made on chickens killed at different times after they had been injected with the various organo-phosphorus compounds. The dose of each compound was either that shown to produce paralysis within 10 days, or, where no paralysis was produced, it was the maximum dose tolerated by the chickens. All the birds except those given TOCP received one or more injections of atropine to suppress the muscarinic symptoms in the acute stages of poisoning. There were no symptoms of acute poisoning after TOCP, but after all the other compounds severe symptoms with collapse of the birds persisted for 12–48 hours. After iso-ompa these symptoms were less than after the other compounds. After administration

TABLE II

NORMAL VALUES FOR AcCh 0·0138 M and BuCh 0·03 M HYDROLYSIS BY CHICKEN BRAIN, SPINAL CORD, SCIATIC NERVE, AND PLASMA

Corrected values for true and pseudo cholinesterases determined as in text. Nervous tissue is expressed as μl. CO_2/g. wet weight/hr. and plasma as μl./CO_2/0·5 ml./min. with the standard deviation

Tissue	AcCh (Total Activity)	True ChE (AcCh) (Corrected)	% True	BuCh (Total Activity)	Pseudo ChE (BuCh) (Corrected)	No. of Animals
Cerebrum	30,066±5,000	29,066±4,840	96	1,393±254	1,093±202	6
Cerebellum	51,313±22,920	50,013±22,700	96·4	1,940±410	1,427±302	6
Cervical spinal cord	3,642±504	3,148±432	89	781±185	530±174	5
Lumbar cord	4,886±724	4,217±251	84	1,154±156	895±63	7
Sciatic nerve	1,655±431	1,287±389	77	501±54	406±38	8
Plasma	8·02±1·52	—	0	—	8·8±1·68	8

of both TOCP and iso-ompa it was found that the true cholinesterase was only partially inhibited; this, therefore, probably accounts for the fewer symptoms.

The doses of the compounds given were as follows:

TOCP: 1 ml./kg. per os, undiluted—paralysis.
DFP: 1 mg./kg. subcut., in 10% ethanol—paralysis.
Mipafox: 20 mg./kg. subcut., in ethanol—paralysis.
TIPP: 8 mg./kg. subcut., undiluted—no paralysis.
Iso-E600: 15 mg./kg. subcut., in ethanol—no paralysis.
Iso-ompa: 300 mg./kg. subcut., in propylene glycol—no paralysis.

Pairs of birds were killed at intervals after administering the compounds, and the activity of the true and pseudo cholinesterase in different parts of the brain, spinal cord, sciatic nerve, and plasma was determined. Only 2 birds were studied after TOCP, and the findings agree with the much larger series published by Earl and Thompson (1952b). Thus true cholinesterase was only slightly inhibited, while the pseudo cholinesterase was reduced to about 20% of its initial activity for at least 10 days. The true cholinesterase of birds treated with iso-ompa was only slightly inhibited, but the pseudo cholinesterase was inhibited by about 70% after 2 days and remained low for at least 6 days. A similar finding with iso-ompa was obtained by Thompson (personal communication). Despite large variations in individual values, the pattern of recovery of cholinesterases after injection of all compounds other than TOCP and iso-ompa was progressive and faster. True cholinesterase was found to be inhibited about 95% 2 hours after injection; it was inhibited 80% 2 days later, and 60% 6 days after injection. Likewise, after 2 hours pseudo cholinesterase was inhibited from 90–100%, about 70% after 2 days, and about 40% after 6 days. Both DFP and mipafox initially inhibited pseudo cholinesterase more completely than the other compounds, although recovery was similar to that after administration of the other compounds. Lumbar spinal cord and sciatic nerve pseudo cholinesterase recovered more slowly than the other pseudo cholinesterases examined.

The figures for the activities of true and pseudo cholinesterase of the cervical spinal cord after each of the inhibitors are given in Table III. The pattern of recovery of chicken cerebrum and lumbar spinal cord after inhibition with demyelinating and non-demyelinating compounds (other than TOCP) is shown in Figs. 1 and 2. There is no clear difference between the rates of recovery after any of the compounds.

TABLE III

CHOLINESTERASE VALUES IN THE CERVICAL SPINAL CORD OF HENS POISONED WITH ORGANO-PHOSPHORUS COMPOUNDS

Activity is expressed as μl. CO_2/g./hr. true cholinesterase in terms of AcCh and pseudo cholinesterase in terms of BuCh (both corrected)

Time After Injection	DFP	TOCP	Mipafox	TIPP	Iso-ompa	Iso-E600
Pseudo Cholinesterase (Normal: 530±174)						
2 hrs.	15		0	150		40
	0		118	213		
1 day	0					
	0					35
2 days	305	0	278	166	122	391
	156		71	46		353
6 ,,	298	0	588	435	409	381
	119		352	312		335
8 ,,			425			
			450			
11 ,,	492					
	529					
True Cholinesterase (Normal: 3,148±432)						
2 hrs.	11		1,170	30		67
	240		950	41		
1 day	610					
	525					330
2 days	450	1,830	1,500	950	3,140	334
	790		1,580	595		356
6 ,,	1,700	3,680	1,890	1,870	2,840	937
	1,430		2,290	1,020		1,395
8 ,,			2,680			
			2,510			
11 ,,	1,810					
	2,330					

Experiments in the Rat in vivo.—Earl and Thompson (1952a) suggested that TOCP failed to produce demyelination in rats because the pseudo cholinesterase of the central nervous system

was insensitive to this inhibitor. Rats were given DFP (0.5 mg./kg.) intravenously. The activity of pseudo cholinesterase in both brain and spinal cord was reduced to zero and returned at a similar rate (40% in 2 days and 60% in 10 days). There were no signs of paralysis, and histologically there was no demyelination.

Discussion

The observations reported here were undertaken as part of an attempt to distinguish between those organophosphorus compounds that do produce demyelination and those that do not. The work was stimulated by the interesting hypothesis put forward by Earl and Thompson (1952a and b) that demyelination might be related to inhibition of the pseudo cholinesterase activity of nervous tissue. A number of compounds, having certain features in common, were chosen for study. Structurally, the compounds were related by the possession of at least two isopropyl groups (except TOCP). Secondly, they all showed preferential inhibition of pseudo cholinesterase rather than true cholinesterase of the chicken when examined *in vitro*. Finally, all the compounds were recognized as being practically "irreversible" inhibitors of cholinesterase in the sense that rates of recovery after inhibition in man and in the rat (Callaway, Davies, and Risley, 1952; Freedman, Willis, and Himwich, 1949; Koelle and Gilman, 1946) approached that of enzyme resynthesis and were indeed very slow when compared to recovery rates after inhibition by E600 or Me-E600 (Aldridge, 1953; Davison, 1953). Such compounds might therefore be expected to produce a profound and a prolonged reduction of pseudo cholinesterase in the hen. It will be seen, however, that in contrast to the prolonged inhibition of pseudo cholinesterase induced by TOCP poisoning all the five remaining compounds produce a similar pattern of changes in the brain, spinal cord, and sciatic

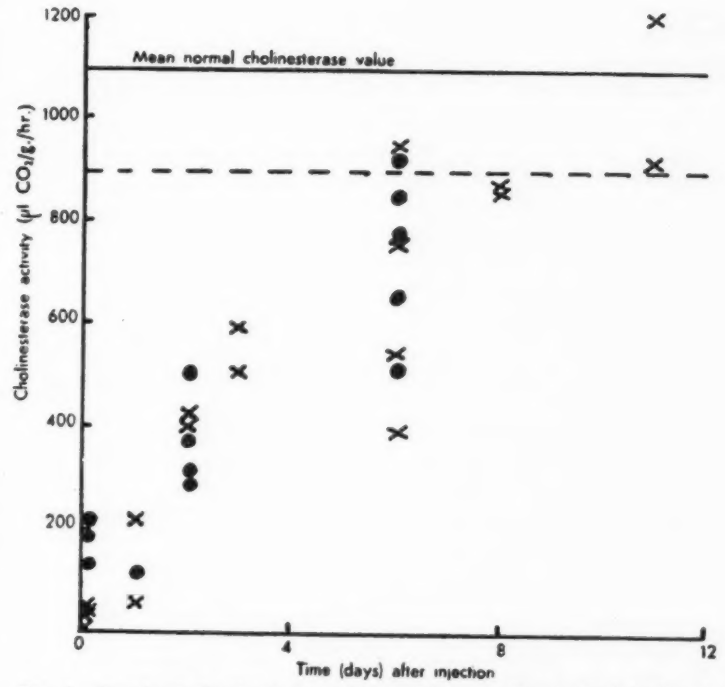

FIG. 1.—Pseudo cholinesterase levels in the chicken cerebrum after injection of organo-phosphorus compounds. Activity is expressed in terms of BuCh, corrected for hydrolysis by true cholinesterase. × demyelinating compounds (DFP, mipafox). ● non-demyelinating compounds (iso-E600, TIPP, iso-ompa). The deviation from the mean is denoted by the broken line.

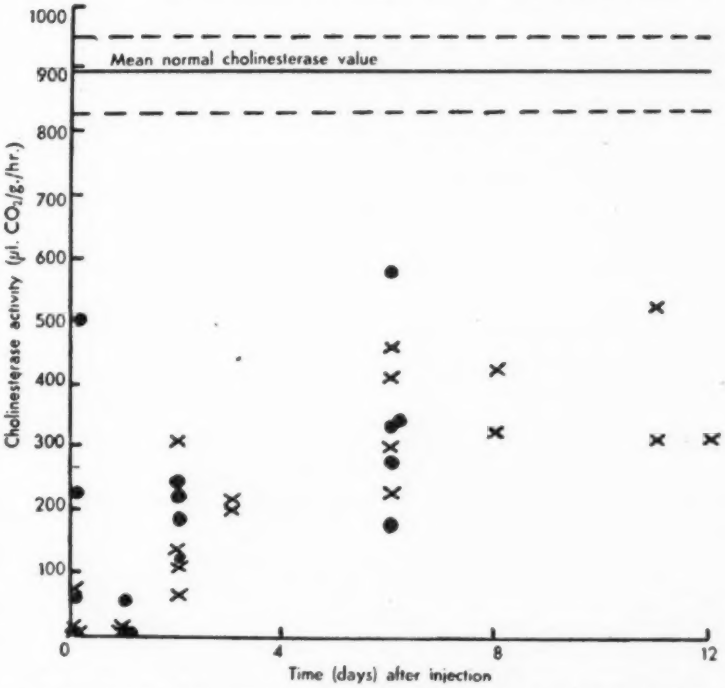

FIG. 2.—Pseudo cholinesterase levels in the chicken lumbar spinal cord after injection of organo-phosphorus compounds. Activity is expressed in terms of BuCh, corrected for hydrolysis by true cholinesterase. × demyelinating compounds (DFP, mipafox). ● non-demyelinating compounds (iso-E600, TIPP, iso-ompa). The deviation from the mean is denoted by the broken line.

nerve cholinesterase levels after the injection of a single dose. There is an immediate lowering of activity to nearly zero within 2 hours, followed by a fairly rapid, but not complete, recovery within 6–8 days. This is faster than the rate of recovery (*in vivo*) of rat's brain cholinesterase, inhibited with DFP (Koelle and Gilman, 1946 ; Freedman, *et al.*, 1949) and considerably faster than the recovery of rabbit brain cholinesterases (Mazur and Bodansky, 1946) after inhibition with DFP. At least two factors may be responsible for the fast recovery of cholinesterase activity in the chicken : (i) reactivation by hydrolysis of the inhibited enzyme, (ii) synthesis of fresh cholinesterase. Since the recovery rate is rapid and indeed faster than that of other species examined it would seem unlikely that fresh cholinesterase was being synthesized so quickly. Further, the rates of return of cholinesterase activity in plasma and brain are similar, although plasma proteins are known to regenerate more quickly than in other tissues (Sprinson and Rittenberg, 1949).

It may therefore be concluded that a prolonged inhibition of pseudo cholinesterase activity of nerve tissue of the central nervous system and sciatic nerve (e.g. TOCP) is not a prerequisite for the production of paralysis. Paralysis is easily produced by a single dose of DFP or mipafox, but the pseudo cholinesterase is completely inhibited for only a few hours, after which recovery is progressive. An indistinguishable pattern of change is also produced by other compounds which do not produce demyelination or paralysis.

Further, demyelination is not produced in rats either by TOCP (Myers and Mendel, 1952) or by DFP, despite complete inhibition of the pseudo cholinesterase of brain and spinal cord by the latter.

The relationship between inhibition of true and pseudo cholinesterase of nerve tissue and demyelination produced by certain organo-phosphorus compounds remains obscure. All that can be said is that demyelination has not yet been produced by a compound that did not also depress the activity of pseudo cholinesterase, but a depression of activity is not enough.

Summary

1. The effect of the injection of five organophosphorus compounds upon the true and pseudo cholinesterase of the brain, spinal cord, and sciatic nerves of chickens is described.

2. Two compounds produced demyelination and three did not. There was no essential difference in the degree or duration of the inhibition of true and pseudo cholinesterase produced by these agents, and none resembled TOCP.

3. DFP will reduce the pseudo cholinesterase of rats, but does not produce demyelination.

4. These observations do not support the theory that prolonged depression of pseudo cholinesterase is an essential prerequisite of demyelination in fowls.

My thanks are due to Dr. J. M. Barnes for his advice and help in the injection of the birds, and also to Miss J. I. Wheatley for technical assistance.

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ALI-ESTERASE INHIBITORS AND GROWTH

BY

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The family of enzymes with which we are concerned in this paper were formerly known as esterases and often confused with lipases. However, since they are incapable of splitting ordinary fats and act preferentially on simple aliphatic esters such as tributyrin, methyl butyrate, and triacetin, they have more recently been renamed ali-esterases (Richter and Croft, 1942). Unlike the cholinesterases, they do not hydrolyse esters of choline. Furthermore, they can be distinguished from the cholinesterases by their insensitivity to inhibition by eserine and prostigmine (Easson and Stedman, 1937; Mendel and Rudney, 1943).

Ali-esterases have been found in higher and in lower forms of life—in animal tissues, micro-organisms, and plants; there is probably no cell which does not contain this type of enzyme. Nevertheless, the physiological functions and the natural substrates of the ali-esterases are still unknown.

The first selective inhibitor of ali-esterases was found in the course of investigations concerned with the function of the pseudo-cholinesterase (Mendel and Rudney, 1943). It was noticed that tri-o-cresyl phosphate (TOCP), a selective inhibitor of the pseudo-cholinesterase in rats (Mendel and Rudney, 1944), is an even more potent inhibitor of ali-esterases in this species (Mendel and Mortimer, 1944; cf. Mendel and Myers, 1953). The ali-esterases (tributyrinases) of serum, liver, and many other tissues can be inhibited *in vivo* almost completely without noticeable ill effects, and such ali-esterase inhibition over a period of many weeks does not affect the well-being or growth of young rats. This discovery raised the question whether, in the course of evolution, the ali-esterases might have lost much of their importance for the animal kingdom, while retaining their essential role in the metabolism of lower forms of life—of viruses, micro-organisms, fungi, plants, and malignant cells. Should this prove true, then ali-esterase inhibition might be a means of interfering selectively with the metabolism and possibly with the growth of invading micro-organisms and other parasitic cells.

A beginning was made in 1944 to test this hypothesis with TOCP on tubercle bacilli (Mendel and Fraser, 1944), which contain large amounts of ali-esterase (Mendel and Hawkins, 1944). The experiments at that time were only a partial success, because the inhibitory action of TOCP, according to our recent investigations, seems to be too slow to keep pace with the formation of new esterase in rapidly multiplying cells. Nevertheless, the idea continued to exert its fascination and has now led to experiments in which it has been found possible, with more potent ali-esterase inhibitors, to arrest the growth of germinating seeds and tubercle bacilli and to inhibit completely the growth of malignant cells without affecting the growth of normal cells in the same tissue culture.

Ali-esterase Inhibitors

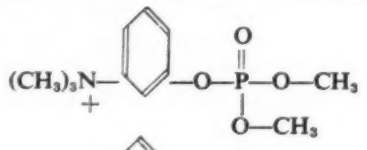
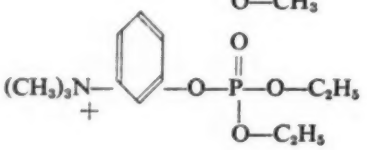
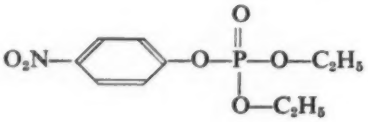
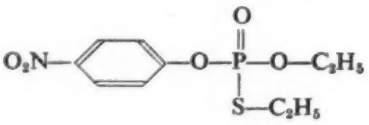
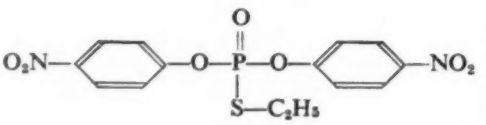
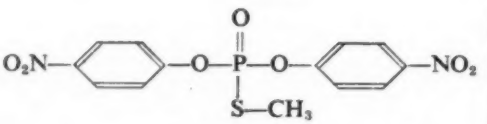
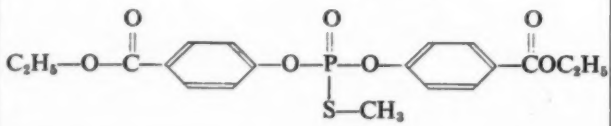
The ali-esterases of sera have recently been divided into two groups by Aldridge (1953a). The results of our experiments support this classification, which might be extended to include similar enzymes found in tissues. The A-type ali-esterase is not inhibited by phosphate tri-esters (Aldridge, 1953a; cf. Myers and Mendel, 1949; Mendel and Myers, 1953), presumably because these esterases are capable of hydrolysing phosphate esters without being inhibited in the process (Aldridge, 1953b). The B-type ali-esterases are inhibited by very low concentrations of tri-o-cresyl phosphate, di-isopropyl fluorophosphonate (DFP), and diethyl p-nitrophenyl phosphate (E600, paraoxon) (Mendel and Mortimer, 1944; Mendel and Myers, 1953; Myers, 1952; Aldridge, 1953a). To these two types of ali-esterase we might add a third, which can only be inhibited by comparatively high concentrations of the phosphate tri-esters mentioned above. An esterase of this kind, which we

might designate as the C-type, is found in mammalian brain (Mendel and Myers, 1953).

The ali-esterases found in lower forms of life often resemble these mammalian ali-esterases in their susceptibility to inhibitors and in their substrate specificity. Yeast cells, for example, contain an A-type esterase, and a B-type ali-esterase has been found in a hepatoma of the mouse; the ali-esterases in mycobacteria, sarcoma, carcinoma, and lymphosarcoma, and the related esterases in

TABLE I

INHIBITION OF THE ALI-ESTERASE (TRIBUTYRINASE) AND TRUE CHOLINESTERASE ACTIVITIES OF RAT BRAIN HOMOGENATE BY ORGANIC PHOSPHATE ESTERS

Compound No. and Designation	Structure	Molar Concentration Causing 50% Inhibition		Ratio of 50% Inhibitory Concentrations: Ali-E/ True ChE
		Ali-esterase	True Cholinesterase	
(1) 3-0412 ..		4×10^{-3}	8×10^{-9}	5×10^{6}
(2) 3-0340 ..		5×10^{-3}	1×10^{-7}	5×10^{4}
(3) E600 ..		$1{\cdot}4\times10^{-7}$	$1{\cdot}4\times10^{-8}$	10
(4)		4×10^{-7}	3×10^{-7}	1·3
(5)		$1{\cdot}6\times10^{-6}$	4×10^{-6}	0·4
(6)		8×10^{-7}	1×10^{-5}	0·08
(7) E 1907..		1×10^{-6}	1×10^{-5}	0·10

The esterase activities were measured manometrically by the Warburg technique at 37° C. and *p*H 7.4, using 0.03 M acetyl-β-methylcholine as a specific substrate for the true cholinesterase (Mendel, Mundell, and Rudney, 1943) and 0.2% tributyrin as substrate for the ali-esterase. The phosphate esters were incubated with a 2% suspension of rat brain for 30 min. before the substrate was added.

Compounds 1 and 2 were synthesized by Roche Products, Ltd., England; compounds 3, 4, 5, and 6 by Professor J. A. A. Ketelaar and Mr. H. R. Gersmann, of the University of Amsterdam, Holland; and compound 7 by Dr. G. Schrader, Elberfeld, Germany.

seeds, resemble more the C-type ali-esterase of mammalian brain. Although TOCP is very suitable as a selective inhibitor of the B-type ali-esterase of serum and liver, intestinal mucosa, kidney, and testis *in vivo* (Mendel and Mortimer, 1944; Mendel and Myers, 1953; Myers and Simons, 1953), it has proved inadequate in the present investigation because the esterases of bacteria, seeds, and tumour cells cannot be inhibited rapidly and effectively enough by TOCP. One of the first problems was, therefore, to find for the C-type ali-esterase a more potent inhibitor, which would not at the same time inhibit the true cholinesterase.

The ali-esterase (tributyrin-esterase) of rat brain homogenate was used as a model of the C-type esterase in these experiments; this has the advantage that the inhibitory action of various compounds on the true cholinesterase and on the ali-esterase can readily be determined with the same tissue preparation under identical experimental conditions. It had been observed that DFP is capable of inhibiting various types of esterase (Webb, 1948), and we found that DFP also inhibits the ali-esterase of brain in a concentration of 10^{-5} M. To determine the structural properties which might be expected to characterize a selective inhibitor of ali-esterases, we studied a series of organic phosphate esters, the most interesting of which are shown in Table I. Within this series, the ratio of inhibitory activities towards ali-esterase and true cholinesterase ranges from 5 million to 0.1; it appears that the selectivity of these inhibitors is largely determined by the same factors which determine the selectivity of reversible inhibitors such as prostigmine and the selectivity of esterase activity towards various substrates (Myers, 1953a, 1953b).

The greatest inhibitory effect on the ali-esterase was observed with E600 (No. 3); this compound still inhibits the cholinesterase somewhat better than it inhibits the ali-esterase, and consequently it is not suitable for experiments on ali-esterase inhibition *in vivo*. Further alterations in the structure of the phosphate molecule have led to compounds (Nos. 6 and 7) which inhibit the ali-esterase of brain in one-tenth of the concentrations which inhibit the true cholinesterase. These compounds are not stable in aqueous solution, and the inhibition *in vivo* after a single injection lasts only for a few hours (Myers, Mendel, Gersmann, and Ketelaar, 1952). Before we can attempt to inhibit the growth of pathogenic cells in an animal, we shall need a more selective ali-esterase inhibitor which gives a longer-lasting inhibition. However, we know now that it is possible to alter the selectivity of an inhibitor over a very wide range, and we can expect to obtain a greater selectivity towards ali-esterase by following up the results given in Table I.

Most of the experiments on the growth of bacterial and malignant cells *in vivo* have been carried out with E600 as inhibitor of the ali-esterase activity. Not only is E600 the most potent of these inhibitors against the C-type ali-esterase, but it is also one of the most stable compounds of this kind. In contrast to the cells of normal mammalian tissues, the malignant and the bacterial cells investigated contain either insignificant amounts of cholinesterase or none at all; moreover, it would not be expected that any cholinesterase present would be concerned with processes of cell growth. Thus the inhibitory action of E600 on cholinesterase need not be taken into account in experiments on cell growth *in vitro*.

Esterase Inhibitors and the Growth of Germinating Seeds

The esterases of seeds resemble the ali-esterases of mammalian tissues in their ability to hydrolyse simple aliphatic esters and in their insensitivity to eserine, but may differ in other respects (Jansen, Jang, and MacDonell, 1947). However, since the acetylesterase of wheat can be selectively inhibited by organic phosphates (Jansen, Nutting, and Balls, 1948), it was of interest to determine whether the esterase inhibitors would have any effect on the germination and growth

TABLE II

EFFECT OF ESTERASE INHIBITORS ON THE GERMINATION AND GROWTH OF OAT SEEDS

Inhibitor	Conc. (mg.%)	Effect on Growth of Seeds			Esterase Activity *in vitro* (% of Control)
		% Which Germinated	% Which Grew	Average Weight of Shoot Plus Root (mg. per Seed)	
E600 ..	0	79	75	103	100
	1	71	62	71	84
	5	73	38	19	49
	10	56	25	8	32
	25	0	0	0	12
E605 ..	0	88	80	102	100
	10	76	50	26	48
	25	72	29	7	19
DFP ..	0	81	75	97	100
	1	90	80	95	98
	5	80	67	75	95
	10	73	59	50	74
	25	69	22	14	52

The effect of the esterase inhibitors on the growth of the seeds was usually determined after 6 days' incubation; all values given are calculated on the basis of the *total* number of seeds used in each experiment.

Esterase activity was measured manometrically by the Warburg technique at 25° C. and *p*H 7.4 with 0.2% tributyrin as substrate: a 2% homogenate of oat seeds was used.

of seeds. In these experiments, wheat (*Triticum vulgare*) and oat (*Avena sativa*) seeds were first soaked for 24 hours in tap water with and without the inhibitor; they were then placed on filter paper, moistened daily with fresh solutions of the same kind, and allowed to germinate in a dark room for three days at about 13° C. and then at 20° C.

The effects of E600, a thiophosphate analogue (E605), and DFP on the growth of oat seeds and on the esterase activity are summarized in Table II. It can be seen that the growth of the germin-

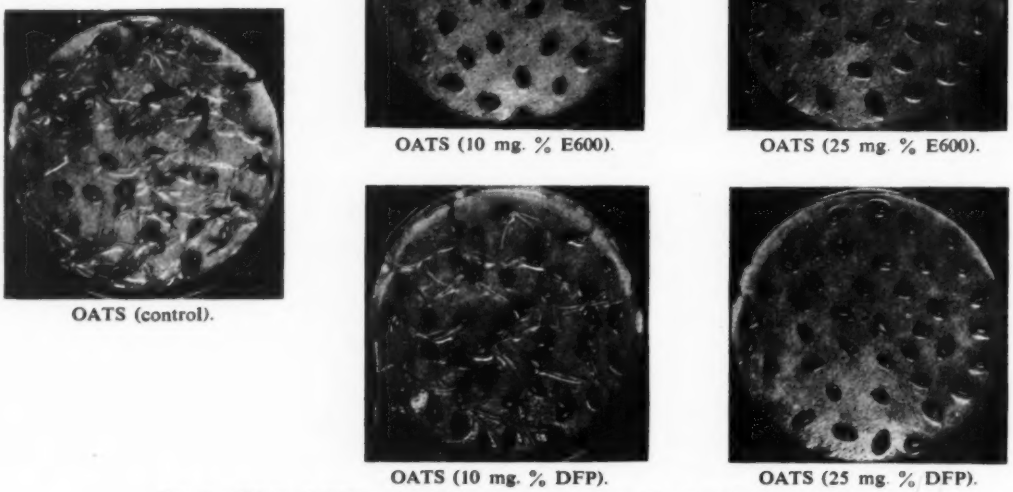


FIG. 1.—Effect of E600 and DFP on the germination of oat seeds after 6 days.

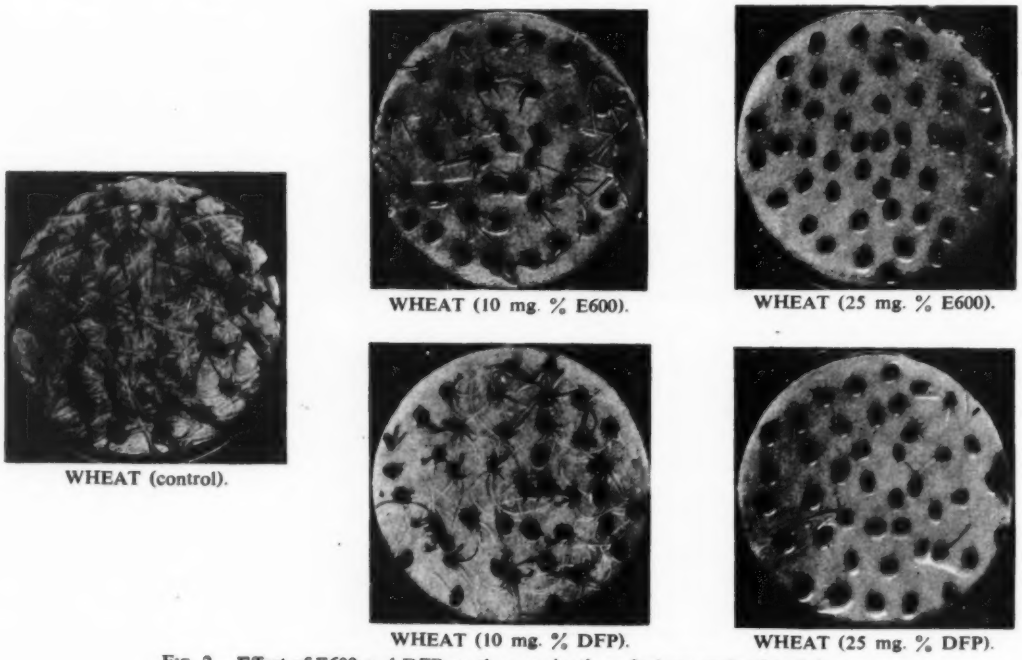


FIG. 2.—Effect of E600 and DFP on the germination of wheat seeds after 6 days.

ating seeds is delayed by all three phosphate esters in concentrations which partially inhibit the esterase activity and that the germination can be completely prevented with a concentration of inhibitor sufficient to inhibit the esterase activity by about 90%. The inhibition of the esterases by E600 is readily reversible, and it was found that the inhibition of growth is also abolished when the inhibitor solution is replaced by tap water.

A marked difference between the esterases of wheat and oat seeds was observed. The esterase of wheat hydrolyses triacetin and nitrophenyl acetate, but has little activity towards tributyrin (cf. Jansen, Nutting, and Balls, 1948); this acetylesterase is about as sensitive to inhibition by DFP as it is to inhibition by E600. The esterase of oats, on the other hand, hydrolyses tributyrin most rapidly, has little activity towards triacetin, and is more sensitive to E600 than to DFP. A correlation with these results was seen in the effects of these inhibitors on the germination and growth of the seeds. Germination and growth of oat seeds are considerably more sensitive to E600 than to DFP (Fig. 1); the shoots of the wheat seeds were about equally sensitive to both inhibitors, while the roots were usually more sensitive to E600 (Fig. 2). These results all substantiate the hypothesis that the inhibition of growth of the germinating seeds by organic phosphate esters is due to inhibition of the esterase activity.

Ali-esterase Inhibitors and Growth of Mycobacterium Tuberculosis

Most of these experiments were carried out with the strain *M. tuberculosis t. humanus* H37RV grown at 36–37° C. in a Dubos medium containing albumin from human plasma. After inoculation with a small amount of a well-grown culture, growth of the bacilli produced a turbidity of the medium within 3–4 days. All experiments were done in duplicate and the results were practically always identical.

The growth of the tubercle bacilli was inhibited by E600 in concentrations as low as 0.05 mg. % (0.5 μg./ml.; ca. 2×10^{-6} M) (Table III). The inhibition of growth was reversible, indicating that the inhibitor was not acting as a non-specific protoplasmic poison. The higher the initial concentration of E600 the longer was the period during which growth was prevented; in some experiments with 1 mg. % E600 the bacilli only started to grow after a period of six weeks. However, once growth had reached a visible level, it continued at the same rate as had been observed in the controls, regardless of how long it had been inhibited previously. The cessation of the inhibitory effect after various periods of time is probably due to the breakdown of E600 and the reappearance of ali-esterase activity. It is known that E600 decomposes slowly in aqueous solution; according to the data of Aldridge and Davison (1952), the original concentration would decrease to one-tenth in about 40 days in a phosphate buffer of *p*H 7.6 at 37° C. However, E600 breaks down much more rapidly in the Dubos medium than in a phosphate buffer of the same *p*H; it appeared that its hydrolysis was catalysed by the albumin in the Dubos medium (cf. Aldridge, 1953b) so that the concentration of E600 decreased to one-tenth in 5–7 days.

Why the inhibition of growth considerably outlasts the presence of the inhibitor in the medium has not yet been fully explained. In any case, the prolonged effect of E600 is not due to breakdown products of the inhibitor which, as we have ascertained, do not influence the growth of tubercle bacilli when present in the same molar concentration as E600; nor did we observe an effect with tri-p-nitrophenyl phosphate—a compound which is much more labile than E600 but a very poor esterase inhibitor.

A few experiments were done with other strains of *M. tuberculosis t. humanus*, one of which was resistant to 100 U. of streptomycin. All of these strains were equally or slightly more sensitive to the action of E600 in the Dubos medium. An experiment was also done according to the method of Price; it was found that the growth of tubercle

TABLE III

EFFECT OF ESTERASE INHIBITORS ON CULTURES OF TUBERCLE BACILLI (H37RV) IN MEDIUM OF DUBOS AT 37° C

Days after Inoculation	E600 (mg.%)						Days after Inoculation	DFP (mg.%)			
	0	0·01	0·05	0·1	0·3	1		0	1	5	20
3	+	+	−	−	−	−	4	+	−	−	−
4	+	+	+	−	−	−	5	+	−	−	−
5	++	++	+	−	−	−	6	+++	+	−	−
6	+++	+++	++	+	−	−	8	++++	+++	+	−
8	++++	++++	++++	+++	−	−	12	++++	++++	++++	−*
12	++++	++++	++++	++++	++	−					
16	++++	++++	++++	++++	++++	++					

* Subculture in Dubos medium without DFP ++ after 22 days.

TABLE IV

EFFECT OF E600 ON CULTURES OF TUBERCLE BACILLI IN THE MEDIUM OF DUBOS AT DIFFERENT TEMPERATURES

Days after Inoculation	*M. tuberc. t. humanus*						*M. avium*		
	36° C. E600 (mg %)			38·5° C. E600 (mg.%)			38·5° C. E600 (mg.%)		
	0	0·05	0·1	0	0·05	0·1	0	0·1	1·0
5	+	−	−	+	−	−	+++	++	+
6	++	−	−	+++	−	−	++++	++++	+
7	+++	+	−	++++	+	−	++++	++++	++
9	++++	+	−	++++	+++	++			
13	++++	++	+	++++	++++	++++			

bacilli in sputum smears on glass slides in haemolysed human blood was strongly inhibited for several days by 1 mg. % E600.

The effects of E600 on the growth of tubercle bacilli were not altered significantly by variations in the *p*H of the medium between 6 and 7.5, nor did the age of the culture and the number of bacilli used for inoculation influence the results appreciably. However, E600 had a stronger inhibitory effect on the growth at 36° C. than at 38.5° C. Since tubercle bacilli multiply somewhat faster at 38.5° than at 36° C., a more rapid inhibition of the ali-esterase, and thus a higher concentration of E600, should be required at 38.5° C. in order to keep pace with the rate of esterase synthesis at the higher temperature. This suggestion is supported by the finding that higher concentrations of E600 were necessary to inhibit the more rapid growth of avian tubercle bacilli (Table IV), and that the even faster growth of saprophytic mycobacteria at 37° was not affected by 1 mg. % E600, while their slow growth at 22° could be partially inhibited by this concentration.

There are some facts which cannot be readily explained as yet: The inhibition of growth by E600 was potentiated by the addition of 2% glycerol to the Dubos medium, and E600 was considerably less active when Tween 80 was omitted from the medium; these results may be due to a change in the permeability of the bacilli to the phosphate ester.

TABLE V

INHIBITION OF ESTERASE ACTIVITY OF TUBERCLE BACILLI BY E600 AND DFP AT 37° C.

Conc. E600 (mg.%)	*p*H		Conc. DFP (mg.%)	*p*H	
	Initial	After 30 min.		Initial	After 60 min.
0	7·0–7·1	6·2	0	7·0–7·1	6·0
0·01	7·0–7·1	6·4	0·05	7·0–7·1	6·1
0·03	7·0–7·1	6·6	0·2	7·0–7·1	6·2
0·1	7·0–7·1	6·8	1·0	7·0–7·1	6·9
0·3	7·0–7·1	7·0	5·0	7·0–7·1	7·0

E600 or DFP was added to 2.5 mg. of tubercle bacilli suspended in a volume of 5 ml. After a subsequent addition of tributyrin in a final concentration of 0.1%, the esterase activity was estimated from the decrease in *p*H caused by the hydrolysis of the substrate.

Experiments on the ali-esterase activity of the tubercle bacilli showed that the hydrolysis of triacetin and tributyrin is almost completely inhibited by 0.3 mg. % E600 and partially inhibited by 0.01–0.1 mg. % after 30 min. incubation (Table V). Thus there is a close correlation between the concentrations of E600 which inhibit the ali-esterase activity and the concentrations which prevent the growth of the tubercle bacilli. Analogous results were obtained with DFP; this compound only inhibits the ali-esterase activity of tubercle bacilli in a concentration of about 1 mg. % (Table V), and the same concentration was necessary to exert a bacteriostatic effect (Table III).

An Ali-esterase Inhibitor (E600) and Growth of a Transplantable Mouse Lymphosarcoma in Tissue Culture

As it is essential to study the action of the substance to be tested on both malignant cells and their normal prototypes, most experiments were carried out with cultures of lymph-glands of a healthy mouse and with four different strains of cells all derived from the lymphosarcoma MB (T86157) described by de Bruyn, Korteweg, and Kits van Waveren (1949), de Bruyn 1949, 1950), and de Bruyn and Gey (1952). A brief characterization of the cell strains follows:

Strain I contains lymphoblasts and fibroblasts. The lymphoblasts produce a tumour, whereas the fibroblasts do not.

Strain II contains only fibroblasts which do not produce a tumour.

Strain III originated in one out of several tubes in which the fibroblasts died out, and apparently a change occurred in the lymphoblasts. Early inoculations showed that the altered cells could produce tumours. However, after growing in tissue culture for approximately four and a half months they lost their ability to produce a tumour in originally susceptible mice.

Strain IV is a mixed strain, containing the fibroblasts of strain II and the altered lymphoblasts of strain III.

The strains derived from the lymphosarcoma were maintained in continuous culture for more than five years. The normal lymphoid tissue was grown for

one or two weeks or directly taken from a lymph-gland of a healthy mouse.

The method employed in these experiments was the roller-tube method of Gey. The medium consisted of various proportions of Tyrode's solution, mouse-embryo extract, human-cord serum, and chicken plasma. E600 was used in a concentration of 10 mg. %, which was found to inhibit the ali-esterase of this particular lymphosarcoma almost completely.

Results.—Thus far six series of experiments were carried out. In all of these experiments E600 inhibited the growth of the lymphoblasts of strain I (Fig. 3), whereas no effect could be seen on the altered, non-malignant lymphoblasts of strains III and IV (Fig. 4) and on the lymphoid cells of cultures of normal lymph-glands. Moreover, E600 had no effect on the fibroblasts of strains I, II, and IV, and on the fibroblasts of cultures of normal lymph-glands. Thus only the malignant cells, i.e., the lymphoblasts of strain I, were affected.

It was noticed that E600 breaks down fairly rapidly in the tissue culture medium. However, no difference could be observed between the growth of the cells in the control medium and the growth in a medium containing a breakdown product of E600 (p-nitrophenol) in the same molar concentration as E600. It is very likely, therefore, that the inhibitory effect of E600 on growth is due to the inhibition of the ali-esterases present in the malignant cells.

Discussion

While our investigations on the ali-esterases were in progress, Smith, Worrel, and Swanson (1949) reported that chloramphenicol inhibits the esterase activity of *E. coli*. We were not able to confirm this finding; a different mechanism for the bacteriostatic action of chloramphenicol has meanwhile been suggested by Gale and Paine (1951). We have also tested various other chemotherapeutic agents, e.g., isonicotinic acid hydrazide and nitrofuraldehyde semicarbazone, against the ali-esterase of mammalian tissues and of tubercle

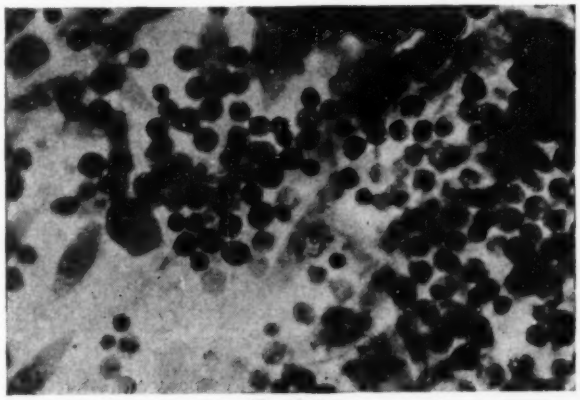


FIG. 3 A

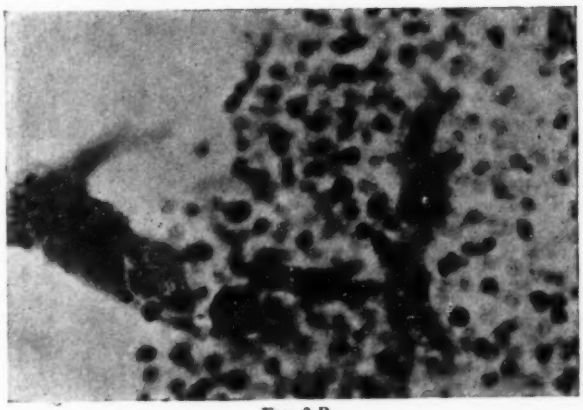


FIG. 3 B

FIG. 3.—(A) Fixed and stained cultures of the malignant Strain I in control medium. Fibroblasts and lymphoblasts in good condition; mitoses. (B) Fixed and stained cultures of Strain I in the medium containing E600. Fibroblasts in good condition; lymphoblasts in very poor condition.

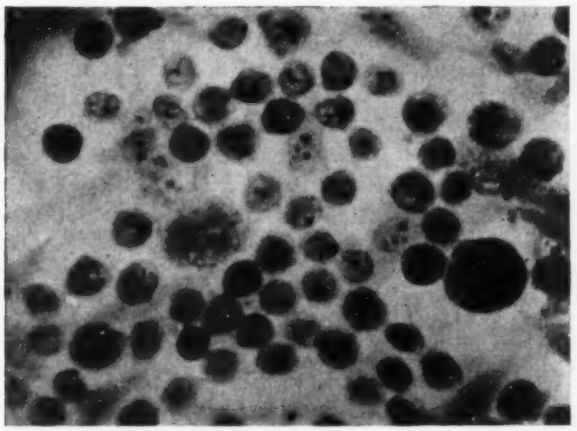


FIG. 4 A

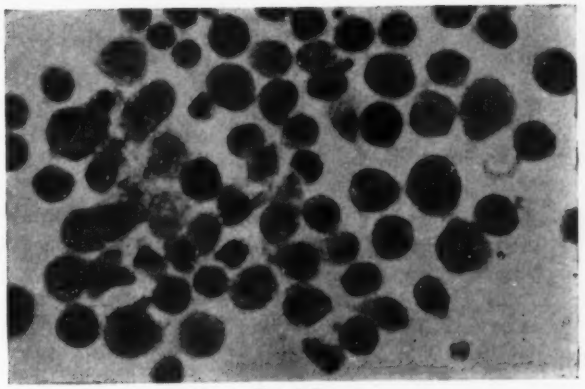


FIG. 4 B

FIG. 4.—(A) Fixed and stained cultures of the non-malignant Strain IV in control medium. Lymphoblasts in good condition; mitoses. (B) Fixed and stained cultures of Strain IV in medium containing E600. Lymphoblasts in good condition; mitoses.

bacilli ; in no case did we observe any significant esterase inhibition by bacteriostatic concentrations. However, our experiments with organic phosphate esters on germinating seeds, tubercle bacilli, and malignant cells appear to support the hypothesis that growth of lower forms of life can be arrested by inhibition of their ali-esterases. We are, of course, aware that our results might be due to inhibition of other hydrolytic enzymes by the phosphate tri-esters (Jansen, Nutting, Jang, and Balls, 1949 ; Hartley and Kilby, 1952). Nevertheless, we consistently find a correlation between the inhibition of ali-esterase activity and the inhibition of growth, and the hypothesis has provided a fruitful basis for our investigations.

Summary

The finding, that an animal is not adversely affected by prolonged inhibition of ali-esterases, led to the hypothesis that the ali-esterases might be enzymes which, in the course of evolution, have lost much of their significance for the animal kingdom, while retaining their essential role in the metabolism of lower forms of life. In order to test this hypothesis the ali-esterases of seeds, tubercle bacilli, and malignant cells have been inhibited by a number of organic phosphate esters. The results of these experiments are as follows:

1. The germination and growth of seeds are inhibited by ali-esterase inhibitors such as diethyl p-nitrophenyl phosphate (E600), diethyl p-nitrophenyl thiophosphate (E605) and di-isopropyl fluorophosphonate (DFP). A close relationship exists between the degree of ali-esterase inhibition and the degree of growth inhibition.

2. The growth of human tubercle bacilli in a Dubos medium is inhibited by E600 in concentrations as low as 0.5 μg./ml. and by DFP in a concentration of 10 μg./ml. Again there is a close correlation between inhibition of ali-esterase activity and inhibition of growth.

3. In tissue cultures the growth of the malignant cells (lymphoblasts) of a mouse lymphosarcoma is inhibited by E600 in a concentration which inhibits the ali-esterase activity. The non-malignant fibroblasts in the same lymphosarcoma, the lymphoid cells and fibroblasts of normal lymph glands, and a non-malignant variation of the lymphoblasts originating from the mouse lymphosarcoma all grow normally in the presence of the concentration of E600 by which the growth of the malignant cells is inhibited.

These results may provide a basis for a new departure towards a rational chemotherapy of tuberculosis and neoplastic disease.

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ANTICONVULSANTS ON ELECTRICALLY STIMULATED METABOLISM OF SEPARATED MAMMALIAN CEREBRAL CORTEX

BY

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Convulsive activity, initiated in the central nervous system of experimental animals by various means, is associated with large biochemical changes. These do not occur when the convulsion is prevented by anticonvulsants. As reported earlier, comparable chemical changes can be brought about in completely separated sections of mammalian cerebral tissues by suitably applied electric potentials (Anguiano and McIlwain, 1951). The present experiments were carried out to find whether the changes induced electrically in the separated tissues were affected by anticonvulsive agents. Such action was found, and further experiments were then carried out to see to what extent the anticonvulsants were distinct from general depressant drugs in their action on the separated, electrically stimulated, cerebral cortex.

The agents studied were chosen to include representatives of the main chemical types of substances which have been used clinically as anticonvulsants, namely: sodium bromide, phenobarbitone, 3:5:5-trimethyloxazolidine-2:4-dione (trimethadione ; Tridione), 5:5-diphenylhydantoin (phenytoin ; Dilantin), and phenylacetylurea (Phenurone). They were compared with the general depressants butobarbitone, chloral hydrate, and urethane.

METHODS AND MATERIALS

Tissue Metabolism.—Guinea-pigs and rats were stunned by a blow on the neck, bled, and the cerebrum removed ; specimens from rabbits were similarly obtained after killing by injecting air into an ear vein. For metabolic experiments, either conical electrode vessels *E* (McIlwain, 1951) were used, or vessels *A* with grid electrodes (Ayres and McIlwain, unpublished ; see Fig. 1). For vessels *E* about 60 mg. of tissue was weighed, chopped (McIlwain and Buddle, 1953), and suspended in saline in the vessel. With grid electrodes, slices 0.35 mm. thick were cut (McIlwain, 1951) and floated into the experimental saline. Slices were trimmed to rectangles about 8×12 mm., drained, weighed (about 35 mg.), fitted to the electrode holders, and these were placed in the vessels already containing saline and absorbing agents, electrical connections tested, the vessels fitted to manometers and shaken at 37° C.

An experiment usually comprised six vessels in which all tissues, whether or not they were subjected to electrical impulses, were between electrodes ; tissues were normally ready for placing at 37° C. 20–30 min. after the death of the animal. Pressure in the vessels was measured each 5 or 10 min. during successive periods, each of which included at least six readings. Experiments normally comprised three or four such periods, and electrical conditions in some of the vessels were changed in successive periods. Salines were those of McIlwain (1951).

Electrical Impulses.—Voltage-time relationships are illustrated in Fig. 1. Condenser pulses were alternating, 100/sec., and of peak voltage and duration

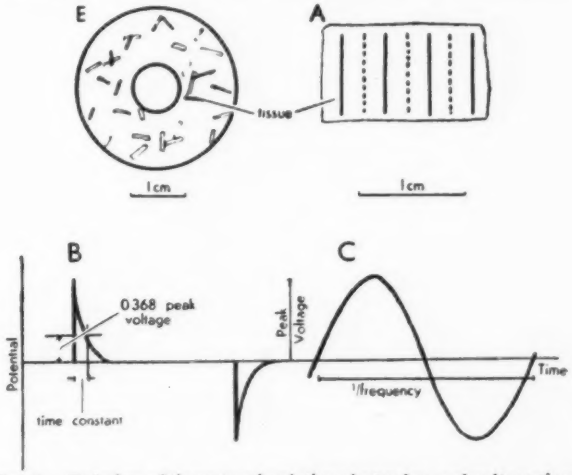

FIG. 1.—Relation of tissue to stimulating electrodes, and voltage-time relationships of impulses employed. A: grid electrodes *A*, the full lines representing wires of a grid connected together above the tissue slices and the dotted lines those also connected together of a grid below the tissue slice; E: concentric electrodes in vessels *E*; B: condenser pulses; C: sine-wave alternating current.

quoted in individual experiments. They were obtained and measured by the instrument described by Ayres and McIlwain (unpublished). Sine-wave alternating current of 50 cyc./sec. was usually derived from mains supply through a transformer; of other frequencies (and also in some experiments of 50 cyc./sec.) it was supplied by a beat-frequency oscillator. The supply voltage and the current through the individual vessels were measured at several times throughout the experiments. The frequency and purity of the sine-wave were determined by displaying the time-voltage relationships with an oscilloscope throughout the experiment.

Materials.—Drugs were of the standards of the *British Pharmacopoeia*, the *British Pharmaceutical Codex*, or of *New and Nonofficial Remedies*. They were dissolved in portions of the experimental saline, and these solutions pipetted into some of the experimental vessels. Tissues exposed to the drug were thus in contact with it as soon as they were placed in the vessels. Trimethadione and phenylacetylurea were obtained from Abbott Laboratories; phenobarbitone, chloral hydrate, diphenylhydantoin, and urethane from British Drug Houses; and butobarbitone from May & Baker. Chloral hydrate is presumably acting partly by virtue of breakdown products (Butler, 1949).

RESULTS

Metabolism of Tissues Unstimulated and Stimulated by Condenser Pulses

Of the metabolic changes induced in cerebral tissues by electrical impulses (McIlwain, 1952), those in respiration are of importance in the tissue's main energy-yielding reactions. In stimulated tissues respiration is affected by general depressants at concentrations below those which affect metabolism in unstimulated tissues (McIlwain, 1953). Table I contrasts such an action of butobarbitone with lack of action in trimethadione or sodium bromide. The concentration of butobarbitone is one found to be attained *in vivo* during its action as a depressant; this concentration has a small effect only on the normal respiration of the tissue, but lowers by some 30 or 40% the respiratory effect of applied impulses. Sodium bromide and trimethadione were examined at concentrations about or much higher than those active *in vivo* as anticonvulsants, but had no effect on respiration either with or without application of condenser pulses.

TABLE I

TRIMETHADIONE, A BROMIDE, AND A BARBITURATE ON RESPIRATION WITH AND WITHOUT CONDENSER PULSES

Results quoted are from individual experiments with guinea-pig cerebral cortex in conical vessels *E*, half of which were not subjected to electrical impulses. Condenser pulses, time-constant 0.4 msec., were applied to others during their second period (peak potential, 10 V) and third period (peak potential, 20 V).

Drug (M)	Respiration in Vessels Without Stimulation (μmol. O_2/g./hr.)			Respiration in Vessels With Stimulation (μmol. O_2/g./hr.)		
	0–30 min.	30–60 min.	60–120 min.	0–30 min.	30–60 min.	60–120 min.
Trimethadione, 0 ..	59	60	60	58	98	116
,, 10^{-3} ..	63	61	61	60	97	112
,, 10^{-2} ..	61	61	61	57	94	115
Sodium bromide, 0 ..	60	59	59	59	102	122
,, ,, 2×10^{-2}	61	58	59	62	100	121
Butobarbitone, 0 ..	65	64	65	62	92	111
,, 7×10^{-4}	61	61	60	58	79	84

In addition to the comparison of Table I, trimethadione has been found to be without effect on the unstimulated respiration of guinea-pig and rat cerebral cortex at concentrations up to 1.33×10^{-2} M, with glucose or glutamate as substrate. Similarly negative results with both substrates were obtained with diphenylhydantoin at 10^{-4} M. These findings are not inconsistent with a previous observation (Taylor, Richards, Everett, and Bertcher, 1950) that trimethadione in extremely high concentrations (0.035–0.14 M) may inhibit respiration of mouse brain slices.

Respiration of Tissues Stimulated by Sine-wave Alternating Currents

Respiration of cerebral tissues is increased by sine-wave a.c. of a wide range of frequencies. The frequency stimulating at minimum voltage is about 50–100 cyc./sec., and stimulation is lost below about 5 or above about 5,000 cyc./sec. In the present experiments frequencies both close to and remote from the optimum were examined. Effects of the anticonvulsants were then discovered, and were found to be dependent on the frequency of the impulses applied.

This is shown in the case of trimethadione in Table II. Here the drug at 2×10^{-4} or 10^{-3} M had little effect on respiration stimulated by 50 cyc./sec. a.c., but it largely prevented a.c. at 500 or 2,000 cyc./sec. from having its usual stimulating effect. Butobarbitone, in contrast, was found to depress respiration stimulated by a.c. at all the frequencies examined, and was thus affecting the metabolic sequelae of a.c. impulses in the same fashion as it acted on those from condenser discharges.

Several drugs were therefore examined with cerebral tissues subjected to a.c. impulses at 50, 500, and 2,000 cyc./sec. The experiments were planned with the following considerations in mind.

(1) Sine-wave a.c. of 2,000 cyc./sec. in the present experimental arrangement increased respiration by not more than about 40%. This value was reached at 3 V, and at 4 or 5 V the respiratory response was no more than at 3 V. Impulses at 2,000 cyc./sec.

TABLE II

TRIMETHADIONE AND BUTOBARBITONE ON RESPIRATION STIMULATED BY SINE-WAVE ALTERNATING CURRENT AT DIFFERENT FREQUENCIES

Results quoted are from individual experiments with slices of guinea-pig cerebral cortex, in tissue-holding electrodes, in vessels *A* containing glucose-phosphate saline. Respiration in each vessel was measured during periods of 40–45 min. in which they were successively without applied impulses, and then with impulses at one of the frequencies chosen. The drugs when present were in the salines before the slices were placed in them.

Drug	Respiration (μmol. O_2/g./hr.)					
	At 50 cyc./sec., 1·3 V		At 500 cyc./sec., 3·5 V		At 2,000 cyc./sec., 3·5 V	
	No Impulses	With Impulses	No Impulses	With Impulses	No Impulses	With Impulses
None	60	99	61	78	69	90
Trimethadione, 2×10^{-4}	58	84	60	65	57	60
,, 10^{-3}	66	96	57	60	66	66
Butobarbitone, 10^{-4}	57	60	—	—	63	66
,, 5×10^{-4}	72	72	—	—	57	66

were therefore applied at 3.5 V. Respiration could be increased to a greater extent by a.c. at 50 cyc./sec., but by choosing a suitable voltage between 1.3 and 2 V a degree of stimulation could be obtained which was comparable to that reached by a.c. of 2,000 cyc./sec., and respiration so increased was used as basis for comparison of the lower and higher frequencies.

(2) Comparison of the effects of the two frequencies is most straightforward if it can be made on the same piece of tissue in the same electrode and vessel. This is possible if the tissue is exposed to the two types of impulse successively. When this was done, the effect of impulses during a second period of stimulation was usually found to be less than their effect during the first (Table III). The fall in effect was most in evidence when the high frequency followed the lower; it was often more pronounced with 2,000 cyc./sec. than with the 500 cyc./sec. quoted in Table III, and persisted when unstimulated periods alternated with the stimulated ones. Nevertheless, because application of two frequencies successively to a given vessel obviated differences in tissue-electrode arrangements which were difficult to standardize exactly, it was always carried out, and Table IV quotes results in the two periods separately. Experiments examining the effect of a given substance were usually carried out in groups of at least two in which vessels and electrodes, with and without the drug being studied, were interchanged.

(3) Comparison of the effect of a given stimulus in several experiments has been found most dependable when the change it induced is expressed as a percentage of the rate in absence of stimulus. In this way experimental variations between individual animals and in weighing and handling the tissue are reduced. The magnitude of this variation can be seen in Table III when different specimens of guinea-pig tissues gave initial unstimulated rates of from 60 to 71 μmol./g./hr. Results have been expressed as per cent change in Table IV. In different experiments of Table IV, differences remain in the effect of a given impulse, in the absence of drugs, in different groups of experiments. This is ascribed to differences in handling the tissue, in electrodes, and in animals over the period of 12 months during which the experiments have been in progress. It is for this reason that values for the effect of impulses in the absence of drugs have been quoted together with the corresponding values in the presence of drugs, in each group of experiments.

The main results of this comparison are given in Table IV. This shows the actions of trimethadione on stimulation at different frequencies to be extremely well differentiated. At 10^{-3} or 10^{-4} M it has no effect with 50 cyc./sec. a.c. during its first period, and a small effect only during the second. In contrast, 10^{-3} M completely prevented

TABLE III

TRIMETHADIONE AND BROMIDE ON RESPIRATION STIMULATED BY LOW AND HIGH FREQUENCY ALTERNATING CURRENT

Experimental arrangement: as in Table II, except where indicated otherwise. Values on each horizontal line are from one piece of tissue observed during successive periods of 40 to 45 min., during which it was alternately unstimulated, exposed to impulses at one frequency, unstimulated, and exposed to impulses at the other frequency. The different periods are given in order from left to right, the first always commencing ten minutes after placing vessels at 37° C.

Drug (M)	Species	Respiration (μmol. O_2/g./hr.) with Impulses (cyc./sec.)						
		None	50	None	500 (or *2,000)	None	50	None
None	Guinea-pig	65	105	60	84	—	—	—
Trimethadione 10^{-3}	,,	60	99	60	66	—	—	—
None	,,	—	75	54	69	60	—	—
Trimethadione 10^{-3}	,,	—	75	54	54	54	—	—
None	,,	—	—	69	84	57	81	—
Trimethadione 10^{-3}	,,	—	—	71	68	68	95	—
None	,,	—	—	—	93	63	85	71
Trimethadione 10^{-3}	,,	—	—	—	69	66	93	54
None	,,	66	108	69	96*	—	—	—
Sodium bromide 3×10^{-3}	,,	60	99	63	69*	—	—	—
None	Rat	—	—	108	136*	112	132	—
Trimethadione 10^{-3}	,,	—	—	110	110*	108	150	—
None	Rabbit	—	—	69	93*	75	90	—
Trimethadione 10^{-3}	,,	—	—	72	68*	72	87	—

TABLE IV

SUMMARY OF EFFECTS OF DRUGS ON RESPIRATORY RESPONSE TO SINE-WAVE ALTERNATING CURRENT

Results are derived from experiments arranged as in Table III. Rates there quoted for trimethadione with guinea-pig give two values for the effect of 50 cyc./sec. a.c. as a first period without drug (e.g., (105–65)/65=62%) and two with drug; and the same number at 500 cyc./sec. Several such percentages (number quoted; values in parentheses refer to second period of stimulation) are averaged in the present Table. Those derived from the period of stimulation which follows soonest after the beginning of incubation are quoted first, and those from second periods of stimulation are given second, in parentheses; when sufficient values are available, each is followed by the standard deviation of the group of values. To obtain probability values *P*, in each experiment the result with drug was subtracted from that without, and the variance of the distribution of these differences in the group of experiments from their mean difference made the basis for determining *t*; *P* was read from a table of *t*.

Drug	Experiments with Current at 50 cyc./sec., 1·3 V or 2* V				Experiments with Current at 500* or 2,000 cyc./sec., 3·5 V			
	No. of Determinations	Increase in Respiration (% of Rate Without Impulses)		*P* for Difference Between Increase Without Drug and Increase With Drug	No. of Determinations	Increase in Respiration (% of Rate Without Impulses)		*P* for Difference Between Increase Without Drug and Increase With Drug
		Without Drug	With Drug			Without Drug	With Drug	
Trimethadione, 10^{-4}	4 (4)	36±17 (29±11)	32±14 (10±5)	>0·9 (0·3)	*4 (4)	49±14 (17±6)	24±8 (5±8)	0·2 (0·5)
Trimethadione, 10^{-3}	*6 (4)	49±10 (27±8)	39±19 (22±10)	0·7 (0·5)	*6 (4) 6 —	35±7 (16±2) 36±4 —	3±6 (1±1) 1±1 —	0·001 (0·001) <0·001 —
Diphenylhydantoin, 10^{-4} ..	4 (4)	46±17 (24±7)	32±5 (9±5)	0·5 (0·2)	*5 (4)	73±8 (18±9)	15±7 (−5±1)	0·01 (0·2)
Diphenylhydantoin, 10^{-3} ..	*4 —	49±19 —	45±19 —	0·9 —	4 —	31±3 —	−4±6 —	0·001 —
Phenobarbitone, 10^{-4}	9 —	44±8 —	45±12 —	>0·9 —	*5 (8)	34±33 (14±6)	29±4 (6±7)	>0·9 (0·7)
Phenobarbitone, $3·5\times10^{-4}$ ..	6 (4)	53±21 (10±1)	41±5 (−2±5)	0·7 (0·05)	*4 (5)	33±10 (34±14)	22±9 (1±6)	0·5 (0·05)
Phenobarbitone, 10^{-3}	— (3)	— (39±17)	— (39±10)	— (>0·9)	3 (2)	28±7 (50)	6±4 (10)	0·05 —
Butobarbitone, 10^{-4}	5 (2)	45±6 (25)	21±8 (8)	0·05 —	3 (4)	30±11 (18±9)	13±10 (4±6)	0·3 (0·2)
Butobarbitone, $3·5\times10^{-4}$ ..	4 (2)	34±10 (20)	12±4 (0)	0·01 —	4 (2)	37±3 (13)	7±9 (−3)	0·02 —
Butobarbitone, 10^{-3}	4 (2)	32±5 (14)	8±3 (−16)	0·001 —	2 (4)	23 (24±3)	−15 (−12±11)	— (0·02)
Urethane, 10^{-2}	2 (2)	27 (29)	28 (10)	— —	2 (2)	32 (15)	11 (12)	— —
Urethane, $2·5\times10^{-2}$ ..	2 (2)	48 (25)	35 (0)	— —	2 (2)	35 (14)	13 (−4)	— —
Urethane, 5×10^{-2} ..	2 (2)	38 (24)	14 (11)	— —	2 (2)	26 (8)	8 (0)	— —
Chloral, 10^{-3} ..	5 (7)	29±4 (21±4)	15±6 (17±6)	0·05 (0·5)	7 (5)	24±6 (13±5)	12±4 (−6±6)	0·1 (0·05)

the effect of 2,000 cyc./sec. a.c., and largely prevented that of 500 cyc./sec. At 10^{-4} M the effect of 500 cyc./sec. was halved. Diphenylhydantoin was comparable, with probably a greater effect at 10^{-4} M.

Phenobarbitone, though showing a similar tendency, was not as specific in its action. Its effects were shown in second rather than first stimulated periods, and were seen at 10^{-4} M or 3.5×10^{-4} M at low and high frequencies. Butobarbitone depressed the effects of low and high frequencies at 10^{-4} M. Its effects increased with increasing concentration and were shown in the first stimulated period to much the same extent as in the second. Urethane and chloral behaved similarly; they showed a possible tendency to inhibit more at the higher frequency, but when this was investigated in the case of urethane by examining a range of concentrations, no clearly differential effect was found.

Trimethadione is seen from Table III to be effective in rat and rabbit tissues as well as in those of guinea-pigs. Sodium bromide selectively depressed at 3×10^{-2} M; results with 10^{-2} M were not decisive.

Phenylacetylurea in some experiments acted selectively, but did not yield consistent results. This was considered due to its low solubility, which did not permit a 10^{-4} M solution to be made in the experimental salines.

DISCUSSION

The present investigations are ones in which actions of anticonvulsants directly related to their central effects have been shown in preparations from the brain *in vitro*. They represent a stage in the analysis of anticonvulsant action which makes it likely that the drugs studied affect the brain directly, without chemical interconversion or other intermediation by the rest of the body. Actions of anticonvulsants previously found *in vitro* have been in peripheral nerve, largely from the frog (see references below). Although this system permits many observations not at present possible in separated cerebral tissues, study of it contributes to knowledge of the actions of anticonvulsant

drugs by analogy rather than by analysis of the situation in which their actions as anticonvulsants are shown. Moreover, trimethadione was not found to affect the frog nerve, but was highly effective in the present study.

Comparison of effective concentrations is important in an analysis such as the present. With *diphenylhydantoin,* the dose 50% effective in antagonizing experimental electroshock is some 0.15 mmol./kg., the therapeutic blood level in man about 0.05 mM, and this concentration acts on frog sciatic nerve (Merritt and Putnam, 1938; Toman and Goodman, 1948; Toman, 1949); action on cerebral tissues in the present experiments is potent at 0.1 mM. *Trimethadione* is 50% effective therapeutically at about 0.3 mmol./kg.; it acts against experimental electroshock seizures at 2.5 mmol./kg., but is without action on frog sciatic at 1 mM (Lennox, 1945; Goodman, Toman, and Swinyard, 1946; Toman, 1952); in the present experiments it is fully active at 1 mM and partly effective at 0.1 mM. *Phenobarbitone* is effective against experimental electroshock at 0.03–0.15 mmol./kg., on peripheral nerve at 1 mM or less (Merritt and Putnam, 1938; Toman, 1952), and is partially active in the present experiments at 0.35 mM. *Phenylacetylurea* has previously been found of too limited a solubility in saline media for *in vitro* observations (Toman, 1952). Effective blood levels of *bromides* as anticonvulsants in man are 10–15 mM, and in the present experiments sodium bromide acted at 30 mM and doubtfully at 10 mM.

Full consideration of possible mechanisms of the present effects is deferred. The different frequencies of applied impulse may excite the same or different nervous elements. Stimulation of the separated cerebral tissue in phosphate buffer and at high frequencies shows resemblance to the situation of "excessive stimulation" found by Toman and Goodman (1948) to be that in which suppression of electrical phenomena by certain anticonvulsants was observed in peripheral nerve. Such suppression in the central nervous system could be understood to play a part in reducing the excessive discharge seen electroencephalographically in the convulsive disorders in which the drugs are effective.

Summary

1. Respiration of separated cerebral tissues when stimulated by sine-wave alternating current at 500 or 2,000 cyc./sec. became sensitive to anticonvulsant drugs. Trimethadione, diphenylhydantoin, and phenobarbitone were effective at 10^{-4} M, and a bromide at 3×10^{-2} M.

2. These substances in the concentrations quoted (and also trimethadione up to 10^{-2} M; diphenyl hydantoin and phenobarbitone up to at least 10^{-3} M) were without comparable effect on respiration of cerebral tissues in the absence of applied impulses. They were also largely ineffective when a comparable degree of respiratory stimulation was induced by sine-wave alternating currents of 50 cyc./sec., or by brief condenser pulses of 100/sec.

3. All the types of stimuli described made the respiration of cerebral tissues more sensitive to the general depressants butobarbitone, urethane, and chloral hydrate.

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THE EVALUATION OF "MYSOLINE"—A NEW ANTICONVULSANT DRUG

BY

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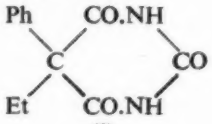
Mysoline* is a new anticonvulsant which was first prepared and evaluated by us and subsequently subjected to clinical trial (Handley and Stewart, 1952). This paper describes some of the methods by which the drug was evaluated.

The drugs commonly used for the treatment of epilepsy generally belong to the same chemical classes as the hypnotics and sedatives, but the hypnotic and anticonvulsant activities do not necessarily go together. In the barbiturate series, phenobarbitone is both a hypnotic and an anticonvulsant, but many other powerful hypnotic barbiturates are almost devoid of anticonvulsant action. The hydantoins, too, have both types of activity, but they are weaker hypnotics than the barbiturates, and in recent years several members of the series with little or no hypnotic action have been introduced as anticonvulsants. These include 5:5-diphenylhydantoin (phenytoin) and 5-ethyl-3-methyl-5-phenylhydantoin (mesantoin, methoin).

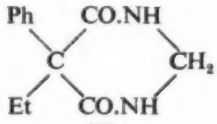
It is unfortunate that the hydantoins in general are rather toxic substances. 5-Ethyl-5-phenylhydantoin (nirvanol), which was introduced many years ago for the treatment of chorea, fell out of use after a short time because of its toxicity. Complaints of toxic side-effects have also been made of both phenytoin and methoin. With the latter this is not surprising in view of recent evidence that in the animal body it is metabolized to nirvanol (Butler, 1952).

The barbiturates are less liable to produce chronic toxic effects than the hydantoins. It is therefore rather surprising that more effort has not been made to obtain non-hypnotic anticonvulsants in this series. The only analogue of phenobarbitone which has found wide use in epilepsy is phemitone (5-ethyl-1-methyl-5-phenylbarbituric acid), which has somewhat less hypnotic and anticonvulsant activity.

Mysoline (5-ethyl-5-phenylhexahydropyrimidine-4:6-dione), formula II, may be considered as a derivative of phenobarbitone, formula I, in which the oxygen in the urea grouping of the barbituric acid is replaced by two atoms of hydrogen. Indeed, among the methods by which it may be prepared (B.P.666,027, W. R. Boon, H. C. Carrington, C. H. Vasey, and I.C.I., 6.2.52) are the electrolytic reduction of phenobarbitone itself, and the catalytic desulphurization of the corresponding 2-thiobarbituric acid.



(I)



(II)

Mysoline is a colourless, remarkably stable, crystalline substance which melts at 281–282° C. It is sparingly soluble in water (60 mg./100 ml. at 37° C.) and in most organic solvents, and, unlike the parent barbiturate, it has no acidic properties.

METHODS

Seizures Induced by Electrical Shock.—A standard convulsion was produced in rats by electrical stimulation, and the effects of drugs on the inhibition of these convulsions were measured.

The method chosen was a modification of that described by Kozelka, Hine, and Grieber (1942) and Alles, Ellis, Feigen, and Redman (1947) in that time rather than current was the variable. The electrical circuit was similar to that used by Tainter, Tainter, Lawrence, Neuru, Lackey, Luduena, Kirtland, and Gonzales (1943). Seizures were induced by passing a 7.5-mA. 50-cycle sinusoidal waveform current by means of padded ear clips. A timing switch was incorporated in the circuit and set so that the maximal duration of the shock did not exceed 10 seconds.

Within a period of 2–7 seconds the normal rat exhibited the characteristic tonic extension of the hind legs. When this was attained the current was switched off and the elapsed time recorded.

Albino rats of both sexes within a weight range of 85–105 g. were used. The initial threshold in terms of the energy required to produce the tonic extensor component of the electrically induced seizure was

*"Mysoline" is the registered trade-mark of Imperial Chemical (Pharmaceuticals) Ltd.

determined for each animal, which then served as its own control. Only those which exhibited the tonic extensor component after a shock intensity of 23–34 milliampere seconds or 600–800 mW. sec. (shock duration 3–4.5 seconds) were selected for anticonvulsant assay. Each assay of a drug at the chosen dose level was carried out on a group of 10 rats.

On the day after the initial threshold determination the drug was administered orally. The maximum dose never exceeded 500 mg./kg.; all doses were given in a volume of 0.5 ml./100 g. body weight. A control group of 10 rats was included in each experiment and was given the same volume of diluent or dispersing agent as was used for the drug under test. Unless otherwise stated, mysoline and other insoluble drugs were administered as an aqueous dispersion in Dispersol OG and LN. This dispersing agent was a mixture of polyglyceryl ricinoleate (OG) and the disodium salt of a disulphonate of dinaphthyl methane (LN).

Seizures were induced at intervals of 1, 3, 6, and 24 hours after dosing and thereafter at 24-hour intervals until the rats returned to within the normal threshold range. The maximum shock intensity delivered to a protected animal was 75 mA. sec. or 2,000 mW. sec. (a 10-second shock).

Anticonvulsant activity was assessed in terms of the dose of drug required to abolish the tonic extensor component of the seizure at this maximal intensity.

Toman, Swinyard, and Goodman (1946), Toman and Goodman (1948), and Goodman, Toman, and Swinyard (1949) have criticized the use of currents of relatively long duration. One of their assay methods is based on the ability of anticonvulsant agents to modify the different phases of the seizure pattern elicited by supramaximal shocks of very short duration (150 mA. for 0.2 sec.). They were able to study these phases independently of the effects of drugs on the energy threshold required to produce maximal seizures. They found that long periods of stimulation concealed the occurrence of drug-modified seizures. Nevertheless, the method as used by us has never failed to show the anticonvulsant activity of clinically recognized anti-epileptic agents or of other compounds reported in the literature. We were not, of course, able to follow the five components of the supramaximal induced seizure described by Toman, Swinyard, and Goodman (1946).

Leptazol (Metrazol) Seizures.—A standard dose of 90 mg./kg. made from a crystalline preparation of leptazol was given intraperitoneally to our own strain of albino rats at 160–180 g. At this dose all untreated rats exhibited maximal seizures followed by collapse, with not less than four deaths out of a group of six. Comparative anticonvulsant activity was assessed in terms of the dose required to prevent maximal seizures and death. Treatment varied from 1–6 hours before leptazol injection. Two groups of 6 treated and 6 control animals were used at each dose level.

TABLE I

THE ACTIVITY OF MYSOLINE AND OTHER ANTICONVULSANTS AGAINST ELECTRICALLY INDUCED SEIZURES IN RATS

Anticonvulsant	Oral Dose mg./kg.	No. of Rats in a Group of 10 in which the Tonic Extensor Component was Abolished						
		Hours After Dosing						Total*
		1	3	6	24	48	72	
Mysoline ..	500	6	10	10	10	10	1	10/10
	200	7	10	10	9	1	—	10/10
	100	5	10	10	5	0	—	10/10
	50	3	9	10	4	—	—	10/10
	20	1	9	10	1	0	—	10/10
	10	1	8	10	0	—	—	10/10
	10	2	7	8	1	—	—	9/10
	5	1	6	5	0	—	—	7/10
Phenobarbitone sodium	20	5	10	10	1	—	—	10/10
	10	3	5	8	1	—	—	8/10
	10	1	6	3	0	—	—	6/10
	5	0	0	2	0	—	—	2/10
5:5-Diphenylhydantoin	200	9	10	10	2	—	—	10/10
	100	9	10	10	0	—	—	10/10
	50	4	5	4	1	—	—	7/10
Methoin (Mesantoin; 5-ethyl-3-methyl-5-phenylhydantoin)	50	9	10	10	0	—	—	10/10
	20	3	9	8	0	—	—	9/10
	10	1	2	0	0	—	—	2/10
Troxidone (Tridione; 3:5:5-trimethyloxazolidine-2:4-dione	500	2	6	2	0	—	—	6/10
	100	0	0	0	0	—	—	0/10
Phenylacetylurea (Phenurone)	100	10	10	10	1	—	—	10/10
	50	8	8	5	0	—	—	9/10
	20	0	4	0	0	—	—	4/10
	10	0	1	0	0	—	—	1/10

* The last column includes all rats which did not exhibit the tonic extensor component at one or more of the test times.

RESULTS

Electrical Seizures.—Table I summarizes the results obtained with mysoline and compares them with those found with phenobarbitone, 5:5-diphenylhydantoin, methoin (mesantoin), troxidone (tridione), and phenylacetylurea (phenurone).

At 5 mg./kg. mysoline was found to be more active than phenobarbitone at the same dose level. The tonic extensor component was abolished in not less than 6 out of 10 animals as compared with 2 out of 10 with phenobarbitone. At 10 mg./kg. mysoline protected 9 or 10 out of 10 as compared with 6 to 8 with phenobarbitone. This activity was not approached by the other anticonvulsants until 50 or 100 mg./kg. was given. At doses from 50 mg./kg. upwards mysoline showed a prolongation of the duration of activity. Mysoline appeared to be slowly absorbed and reached its maximum activity at 3–6 hours after administration at low or moderate dosage. At high dosage (500 mg./kg.) the duration of protection exceeded 48 hours, but there was marked ataxia in rats. Some ataxia was apparent at 250 mg./kg.

A few additional tests were carried out at other time intervals with the object of determining the duration of highest activity. These results at 3 dose levels are summarized in Table II. At 50 and 100 mg./kg. 80–90% of the rats were protected at 6 hours, and this degree of protection persisted until at least the 12th hour after dosing. At 25 hours both groups returned to their normal threshold. At 10 mg./kg. 70% of the rats were protected at one or more periods of the test ; here again the degree of protection afforded by this low dose was apparent at the 12th hour. At 25 hours the activity had gone and all the animals responded within the normal threshold range. Phenobarbitone at 10 mg./kg. is also included in the table for comparison ; 80% of the animals were protected, and this degree of protection lasted for 12 hours. All this group exhibited the tonic extensor component within the normal threshold range at 25 hours.

The activity of the other anticonvulsants listed in Table I was virtually lost at 24 hours at subtoxic doses.

Fig. 1 summarizes the results obtained in measuring the energy threshold in 140 rats. Six groups of 10 served as controls. It will be seen

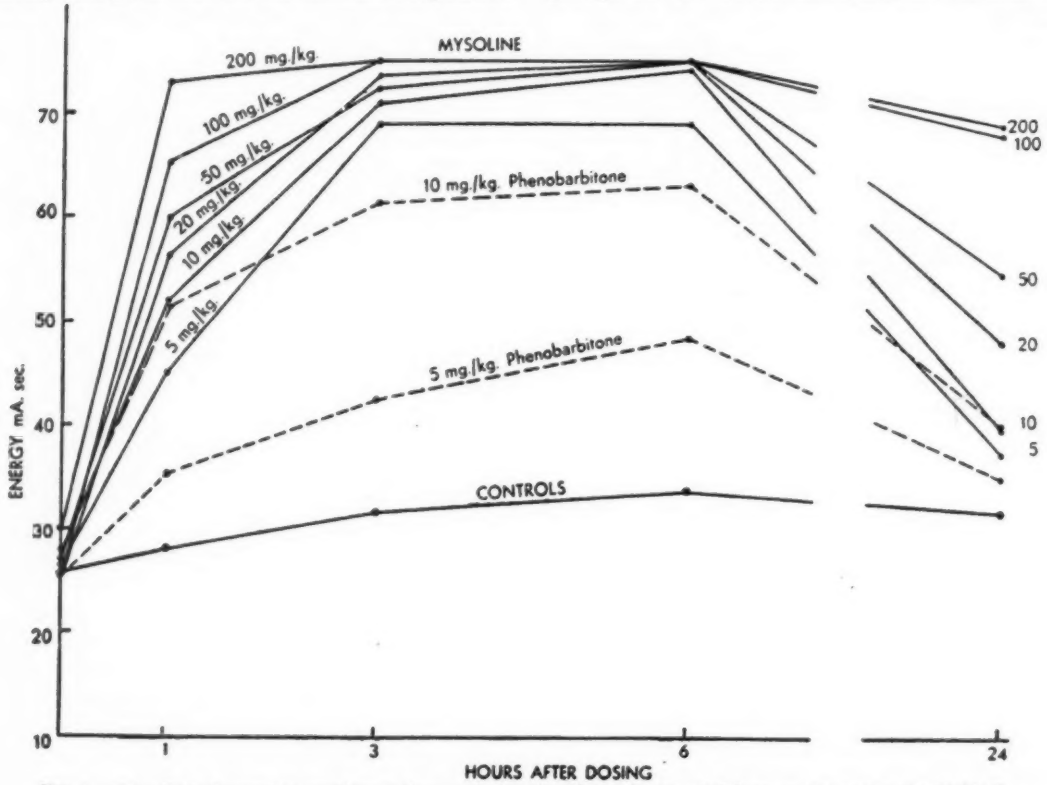


FIG. 1.—The effect of mysoline, continuous lines, and phenobarbitone, interrupted lines, on the energy threshold of electroshock seizures in rats.

TABLE II

DURATION OF PROTECTION AFFORDED BY MYSOLINE AGAINST ELECTRICALLY INDUCED SEIZURES

Anticonvulsant	Oral Dose mg./kg.	No. of Rats in which the Tonic Extensor Phase of the Seizure was Abolished					
		Hours After Dosing					Total*
		3	6	9	12	25	
Mysoline ..	100	—	9	10	9	2	10/10
	50	—	8	9	8	1	10/10
	10	4	7	6	6	0	7/10
Phenobarbitone ..	10	6	7	5	6	0/9	8/10

* The last column includes all rats which did not exhibit the tonic extensor component at one or more of the test times.

that those animals selected for test when the initial threshold was determined remained remarkably constant in terms of the energy required to produce the tonic extensor component of the seizure. It will also be seen that there is a slight rise in threshold in the controls due to the shock regime which levels off at three hours. This increase in threshold, however, lies within the initial selected threshold range.

The elevation of the threshold in groups of not less than 10 rats by mysoline is shown at 6 dose levels, namely 5, 10, 20, 50, 100, and 200 mg./kg. At 5 mg./kg. 60% were protected, but, while the

maximum activity was apparent at 3 hours, a few rats show the beginning of a fall in threshold between the third and sixth hour. At 10, 20, and 50 mg./kg. the threshold rose rapidly during the first 3 hours and reached its maximum at 6 hours. At about 10 mg./kg. all animals were protected and received 2,000 mW. sec. or 75 mA. sec. without exhibiting the end-point. At 200 mg./kg. the threshold had virtually attained its maximum at 1 hour. At 50, 100, and 200 mg./kg. the thresholds were still significantly elevated at 24 hours. The elevation of the threshold by phenobarbitone at 5 and 10 mg./kg. is also shown for comparison.

Mysoline was also given intravenously and intramuscularly in order to determine any modification in the onset and duration of activity. When given intravenously the effects were apparent within the first hour, but after intramuscular injection the effects were somewhat delayed when compared both with intravenous and oral administration. At the third hour, however, there was little to choose between any of these routes. The prolongation of activity was only apparent when 50 mg./kg. was given intramuscularly, there being 100% protection at 24 hours as compared with 40% when 50 mg./kg. was given by the oral route.

The minimal single oral dose of mysoline required to abolish the tonic extensor component of the electrically induced seizure in more than 50% of rats was therefore 5 mg./kg.

Leptazol Seizures.—Fig. 2 shows the results obtained with mysoline and other anticonvulsants in rats given a standard dose of leptazol (90 mg./kg. i.p.). The anticonvulsant was given orally. The period of treatment before the administration of leptazol was governed by the results obtained from the electrically induced seizure experiments. With mysoline and those drugs which were insoluble it was found that a pretreatment period of 4–6 hours was desirable in order to obtain the maximum effect. The effect of a longer treatment period was more apparent at low dosage.

Complete control in terms of the prevention of maximal seizures in every animal in a group was obtained with mysoline at 200 mg./kg. The most active drug in this test was phenobarbitone, there being no maximal seizures at 20 mg./kg. and only 5 out of a group of 12 at 10 mg./kg. with one death. Methoin at 50 mg./kg. also prevented maximal seizures in a similar group.

While at 10 and 20 mg./kg. mysoline was found to be slightly more active than methoin, phenylacetyl urea, and troxidone (tridione), there was little to choose between the protective activity of mysoline, phenurone, and troxidone, all of which were completely effective at 200 mg./kg. At 100 mg./kg. there was 1 maximal seizure with phenurone, 2 with mysoline, and 11 out of 12 including 3 deaths with troxidone. With troxidone the females seemed to be afforded a higher degree of protection against death as compared with males in the same experiment. 5:5-Diphenylhydantoin showed little or no activity in this test at 200 mg./kg.

The minimal single oral dose of mysoline required to prevent maximal leptazol seizures in 50% of rats was found to be 20 mg./kg.

Toxicity of Mysoline

Acute Toxicity in Rodents.—The LD50 in mice was from 600 to 800 mg./kg. In rats the LD50 was 1.5 g./kg. for the 100 g. rat and greater than 2.0 g./kg. for the 200 g. rat. Some ataxia was apparent in rats following a single dose of 250 mg./kg. At 500 mg./kg. this was quite marked except in the 200 g. male, which did not show this effect. Fifty per cent of rats of 100 g. showed hypnosis after a single dose of 1.5 g./kg. Rats of 200 g. or over, particularly males, were more resistant to the onset of hypnosis.

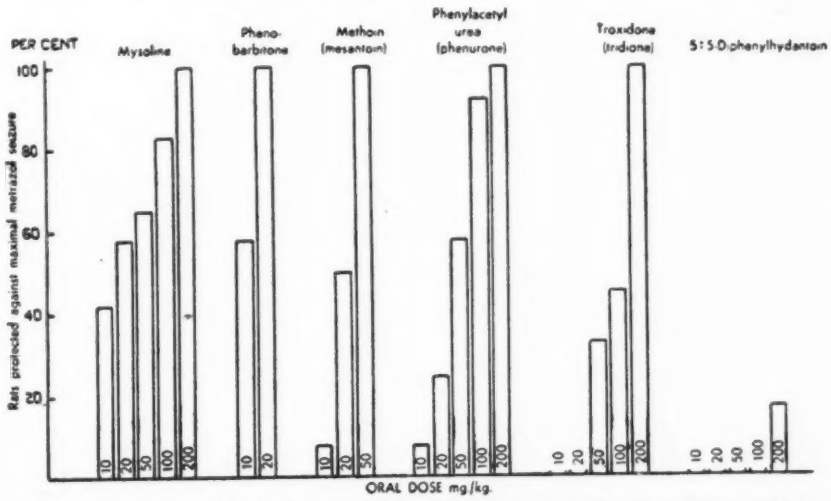


FIG. 2.—The degree of protection afforded by mysoline and selected anticonvulsants against maximal seizures in rats given a standard dose of metrazol, 90 mg./kg. i.p.

Chronic Toxicity in Rats.—Three dose ranges, 125, 250, and 500 mg./kg., were used in groups of 10 male and 10 female rats of 120–150 g.

weight. Twenty doses were given in 4 groups of 5 daily doses over a period of 4 weeks. The rats were not subjected to any treatment over the week-end.

At 125 mg./kg. the first signs of ataxia were noted in the males on the fourth day 3 hours after dosing; this was also apparent in both sexes on the fifth day. On the eighth day, i.e., after the sixth dose, ataxia was noted in 5/10 males. All rats showed mild ataxia on the ninth day. At the beginning of the third week there was no ataxia after dosing, and subsequent dosing during this week produced no more than a trace of ataxia. During the fourth week the males appeared to be more resistant than before. No symptoms were observed in either sex from the 17th to the 20th dose, which ended the experiment. There were two deaths, one female on the fifth day and one male on the 17th day. The females showed an average gain in weight of 46 g. and males 90 g.

At 250 mg./kg. there was some ataxia after the first dose, but it was not until the fourth dose that any marked ataxia was apparent in both sexes. After the sixth dose ataxia was seen in the males, while the females showed milder symptoms. During the third week ataxia did not develop until the 14th dose. In the fourth week, only a trace of ataxia was observed. There was one death in the females during the third week. The females showed an average gain in weight of 41 g. and the males 85 g.

At 500 mg./kg. there was severe ataxia 5 hours after the first dose, and 24 hours later 6/10 females appeared ill. Subsequent doses produced torpor and hypnosis. Six deaths occurred between the fourth and sixth day and another four during the second week. Severe ataxia and torpor persisted throughout the period of the experiment. The surviving four females showed an average weight gain of 28 g. and the six males 69 g.

Growth of Rats.—The growth rate of 191 newly weaned 4-week-old rats from 19 litters was determined over a test period of one week. The rats were then divided into three groups so that each group showed a similar weight gain and contained the same number of rats from each litter. Each group was made up of 25 males and 25 females. Two groups were given 45 doses of mysoline at 100 and 250 mg./kg. respectively over a period of 9 weeks. The third group served as control, received no treatment, and were also weighed daily. The mysoline-treated groups showed a percentage weight gain of 314 at 100 mg./kg., 307 at 250 mg./kg., and the controls 341.

The appearance and subsequent reduction of ataxia observed in the chronic test despite continued dosing were also apparent in the growth trials.

Histological Examination.—On completion of both the chronic toxicity and growth experiments in rats a detailed histological examination was made at all dose levels.

In the chronic toxicity tests, at 25 to 100 times the dose required to protect 60% of rats against seizures induced by electrical shock, there was a suspicion of change in the convoluted tubules which could be attributed to the drug. This damage was more definite in the growth experiment in which rats received mysoline for more than double the time at 100 and 250 mg./kg. There was swelling of the cells of the convoluted tubules, vacuolation of these cells, and desquamation of damaged cells into the tubular lumina. The occasional presence of dividing cells in the tubules suggested regeneration after desquamation. One or more of the above changes were seen in the kidneys of 40% of the rats examined at both dose levels. Many of these changes were seen to a lesser degree in rats given dispersing agent only. However, they were more severe at the higher dose level and can therefore be attributed to the chronic administration of the drug.

The thyroid showed morphological change in the growth experiment in 44% of rats at 100 mg./kg. and 56% at 250 mg./kg. This consisted of a deepening of the usual cubical epithelium to low columnar or even to true columnar, which was sometimes associated with poorly staining colloid and mild peripheral vacuolation. The thyroids of the controls were all within normal limits.

All other organs failed to show any consistent change which could be attributed to the drug.

The above results indicate that a single dose of 100 times the therapeutic dose required to suppress electrically induced seizures in over 50% of rats was necessary to produce definite neurotoxic symptoms, and that with repeated administration over a period of 9 weeks at least 25 times this dose was required to produce any appreciable pathological change.

Toxicity of Mysoline in Monkeys.—Three monkeys were each given mysoline at 50, 100, and 250 mg./kg. respectively by stomach tube once daily in 8 groups of 5 doses on successive days for 8 weeks. The largest dose caused ataxia, which increased with daily dosing and disappeared over the week-end. There were no symptoms at the two lower doses. All three monkeys gained weight

during the course of the experiment. Weekly blood counts were normal.

A further five monkeys were given mysoline by stomach tube at 500 mg./kg. on four successive days. The first dose produced little or no effect. The first signs of ataxia were seen 3 hours after the second dose ; torpor was apparent at 7 hours, and ataxia still evident 24 hours later. The third dose caused three of the monkeys to vomit, two showed hypnosis, and three marked ataxia. Torpor and ataxia were still marked before the fourth dose. Three were killed on the fourth day and two were kept. These latter were still torpid on the fifth day. Ataxia disappeared by the eighth day.

Histological Examination.—The changes seen in the kidneys of the monkey which received 250 mg./kg. for 8 weeks consisted of dilatation of the convoluted tubules with a rather low epithelium with ragged outlines. Many of the convoluted tubules contained granular or eosinophilic material together with an occasional desquamated epithelial cell. There were a few small areas of cellular infiltration around groups of tubules. There was probably some evidence of change in the monkey which received 100 mg./kg. for 8 weeks. At 500 mg./kg. there were some pyknotic nuclei in the medullary tubules, otherwise the kidneys did not differ materially from the control. The changes in the thyroid at 250 mg./kg. and 500 mg./kg. did not differ from those found in the control dosed with dispersing agent. All other organs were within normal limits at all dose levels. In the control monkey which received dispersing agent only, the majority of the convoluted tubules were dilated. There was also some eosinophilic amorphous substance in the lumen of the tubule with an occasional desquamate cell.

These findings were in agreement with those obtained in the rat toxicity trials.

Discussion

The anticonvulsant potency of mysoline compares favourably with phenobarbitone, phenylacetylurea, methoin, 5:5-diphenylhydantoin, and troxidone. This potency, however, is only of clinical significance when the effective dose is considered in relation to the toxic dose. Mysoline has been shown to possess a low toxicity in laboratory animals. It is much less toxic than phenobarbitone, a dose of the order of twenty times that of phenobarbitone being necessary to produce a similar degree of neurotoxicity. If the effective dose of mysoline required to protect 50% of rats in the electrical test, 5 mg./kg., is compared with the LD50, 1.5 to 2.0 g./kg., then the therapeutic ratio is between 300 and 400:1 for a single dose. If the therapeutic ratio is related to the single dose required to produce neurotoxicity, the ratio is then at least 50:1. Therapeutic ratios based on single-dose observations are of little value when considering drugs which have to be taken chronically.

In our opinion, a more realistic figure is that obtained by relating the effective dose to the chronic dose required to produce neurotoxicity, and possibly some pathological change, when administered over a period of at least one or two months. In our experiments the chronic neurotoxic dose is 250 mg./kg., which also gives a therapeutic ratio of 50:1.

A peculiar feature of the chronic trials, including the growth experiment, is the disappearance of ataxia with continued dosing at 250 mg./kg. from the beginning of the fourth week onwards. At double this dose the ataxia persists.

In the leptazol test the effective dose required to prevent maximal seizures in 50% of rats is 20 mg./kg., which gives a ratio of 12.5:1. If, however, the effective dose is taken as that required completely to prevent even submaximal leptazol seizures, then the ratio, based on the single neurotoxic dose, approaches unity.

The ultimate proof of the validity of our methods is only to be found in clinical trial on man. Such a trial has been reported by Handley and Stewart (1952). They confirmed the anticonvulsant activity of mysoline in grand mal epilepsy and its lack of serious toxicity.

At 1.6 g. per day they reported that 4 of their patients (10%) showed transient side-effects such as nausea, dizziness, and mild ataxia at the beginning of treatment. All these symptoms pass off in a few days and treatment was not interrupted. These observations are paralleled to some extent by disappearance of ataxia in rats and dogs even with continued dosage. Nausea was observed in monkeys at a very high dosage, 500 mg./kg. The patients observed by Handley and Stewart have now been under mysoline treatment for two years and have failed to show any blood dyscrasias or other abnormality which could be attributed to the drug. The clinical trials on grand mal epileptics suggest that the toxicity and therapeutic ratio agree more closely with the electric shock than the leptazol observations. The electrically evoked seizure is probably more comparable with human grand mal than the leptazol induced seizure.

The low toxicity of mysoline in experimental animals cannot be attributed solely to low water solubility and lack of absorption. A blood level

of over 20 mg. % has been observed in monkeys, while in rats a blood level of the order of 12 mg.% has been found six hours after a single dose of 250 mg./kg.

Summary

1. The anticonvulsant action of mysoline, 5-ethyl-5-phenylhexahydropyrimidine-4 : 6-dione, against electroshock and leptazol seizures in rats has been demonstrated and compared with phenobarbitone, 5:5-diphenylhydantoin, methoin (mesantoin), troxidone (tridione), and phenylacetylurea (phenurone).

2. A single oral dose of 5 mg./kg. of mysoline was sufficient to abolish the tonic extensor component of the electrically induced seizure in over 60% of rats, while a single dose of 20 mg./kg. of mysoline was required to prevent maximal leptazol seizures in over 50% of rats. This compares favourably with other anticonvulsant drugs.

3. Mysoline was shown to have a remarkably low toxicity in animals, both acute and chronic, whether expressed in terms of the LD50 or as the dose required to produce neurological symptoms or morphological change.

We are greatly indebted to Miss S. Bentley for her most valuable and skilful technical assistance.

We wish to thank Dr. E. Weston Hurst and Dr. D. C. Roberts for the histological examinations.

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A BLOWPIPE DART POISON FROM NORTH BORNEO

BY

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In a recent paper Robinson and Ling (1953) have described the pharmacological examination of a dart poison used by the Pĕnan tribe of North Borneo. Their results showed that the activity of the preparation was due to the presence of cardiac glycosides, the crude material having 20% of the activity of ouabain in the rabbit, and it was suggested that the toxic principle was perhaps antiarin, a glycoside obtained from the upas tree, *Antiaris toxicaria* Lesch (Moraceae) which is widely used as an arrow poison throughout the Malayan archipelago and Indonesia, although its use has not apparently been reported previously from North Borneo. Through the courtesy of Professor J. H. Burn, the remainder of the poison (8.2 g.) was made available to the present author for chemical investigation.

RESULTS

The poison was received in the form of brown resinous lumps, which were powdered. The following method of extraction was found to yield the best results. The powder (5 g.) was digested with water (50 ml.) on the steam bath, when it dissolved readily and almost completely. The solution was acidified with a few drops of 0.1 N-sulphuric acid, filtered with the aid of a little Celite to remove a small amount of suspended matter, and extracted with ten portions of a 2:1 mixture of chloroform and ethanol (750 ml. in all). The combined organic layers were then washed with a little sodium bicarbonate solution and with water and evaporated. A solution of the residue (1.79 g., 36%) in a small volume of aqueous methanol, after standing for some time and seeding, deposited a crystalline solid, m.p. 210–220° C. (162 mg., 3.2%) which was filtered off and after several recrystallizations from the same solvent had m.p. 237–239° C., $[\alpha]_D^{21} = -3 \pm 2°$ (MeOH). This substance gave a red colour in the Legal test and showed the ultra-violet absorption maximum at 218 mμ characteristic of the cardiac glycosides ; also a few mg. with a drop of concentrated sulphuric acid gave a yellow solution, slowly becoming green. These properties are in accordance with those described for α-antiarin.

An authentic sample of α-antiarin, m.p. 238–240° C., kindly provided by Professor Reichstein, showed no melting-point depression when mixed with the present specimen, and the colour reactions and crystalline form of the two were similar.

Further to confirm the identity of the substance with α-antiarin, the benzoate was prepared, which after crystallization from methanol had m.p. 240° C., $[\alpha]_D^{21} = +3 \pm 2°$ (acetone). Doebel, Schlittler, and Reichstein (1948) give m.p. 240–241° C., $[\alpha]_D^{21} = +2.4$ (acetone) for α-antiarin benzoate.

The mother liquors from the crude crystalline antiarin gave an intense Legal test, and must still contain a considerable amount of antiarin or other cardiac glycosides, and it is probable that higher glycosides of antiarin may be present, as di- or triglycosides of cardiac aglycones are usually much more plentiful in plants than are the simpler monoglycosides.

SUMMARY

It has been shown, in agreement with the pharmacological results of Robinson and Ling (1953), that a dart poison used by the Pĕnan of North Borneo contains α-antiarin as an active constituent.

The author is grateful to Dr. R. K. Callow for his interest.

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THE ACTION OF 5-HYDROXYTRYPTAMINE ON PULMONARY AND CARDIOVASCULAR VAGAL AFFERENT FIBRES AND ITS REFLEX RESPIRATORY EFFECTS

BY

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The identification of 5-hydroxytryptamine with serotonin (Rapport, 1949) made it of interest to examine further its respiratory effects in cats. Reid and Rand (1951) have reported that 5-hydroxytryptamine may cause apnoea in cats, and Douglas and Toh (1952) found that, in dogs, this compound stimulated the chemoreceptors of the aortic arch and carotid body. Phenyl diguanide also has been shown to cause a reflex inhibition of the respiratory movements, abolished by vagotomy, in cats. This is due to an action on receptors in the lungs (Dawes, Mott, and Widdicombe, 1951). Phenyl diguanide also stimulates the chemoreceptors of the aortic arch and carotid body in dogs (Dawes, Mott, and Widdicombe, 1952). Further, this compound stimulates certain vagal afferent nerve fibres which are normally quiescent and whose mean conduction velocity is 6 m./sec. (Paintal, 1953c). This low conduction velocity corresponds with the low temperature (below 3° C.) to which the vagi must be cooled to block the respiratory reflex. It seemed possible that the apnoea caused by 5-hydroxytryptamine in cats might be a reflex due to the stimulation of those receptors which are stimulated by phenyl diguanide and other amidine compounds. Reid (1951) found that the apnoea caused by tryptamine in cats was abolished by vagotomy, but Page (1952) states that the respiratory effects of 5-hydroxytryptamine are not modified by vagotomy.

METHODS

The experiments to be described were performed on cats of 2.1–3.3 kg. in weight, anaesthetized with chloralose (60–80 mg./kg. body weight).

The technique of dissecting single units of the vagus and determining their conduction velocity has been described elsewhere (Paintal, 1953b). The right venous pressure was recorded with a semirigid catheter passed down the external jugular vein and connected to a saline-filled capacitance manometer. Intrapleural pressure was recorded with a wide-bore needle connected to a mirror membrane manometer. The electrocardiogram (lead 1) was recorded, using a condenser-coupled amplifier and cathode-ray oscilloscope. Various kinds of afferent fibres were dissected and, after identification, 5-hydroxytryptamine was injected intravenously into the femoral vein while the activity of the fibre was being recorded. The phenyl-diguanide-sensitive fibres were isolated by a method to be described elsewhere (Paintal, 1953c), and the effect of 5-hydroxytryptamine on them was tested by injecting the drug about 15 minutes after the injection of 100 μg. phenyl diguanide.

Carotid blood pressure was recorded by a mercury manometer. The left auricular appendage was cannulated in six cats, and the vagi prepared for cooling in three cats; the respiratory movements were recorded by a body plethysmograph (Dawes, Mott, and Widdicombe, 1951), and the 5-hydroxytryptamine was injected intravenously into a jugular vein. All doses are in terms of 5-hydroxytryptamine. The depressor effect of this compound was found to be very great. In order to obtain apnoea without too much cardiovascular disturbance, it was necessary to give atropine 1 mg./kg. body weight.

RESULTS

Localization of Receptor Area.—In six cats equal doses of 5-hydroxytryptamine were injected alternately intravenously and into the left atrium, and the effects on the respiratory movements observed. In four cats 45–90 μg. of 5-hydroxytryptamine caused apnoea (almost always in the expiratory position) when injected intravenously, but not when injected into the left atrium; this inhibition of the respiratory movements was abolished by vagotomy (Fig. 1). In a fifth cat, on one occasion, 22.5 μg. of 5-hydroxytryptamine, injected into the left atrium, failed to cause inhibition of respiratory movements, although this

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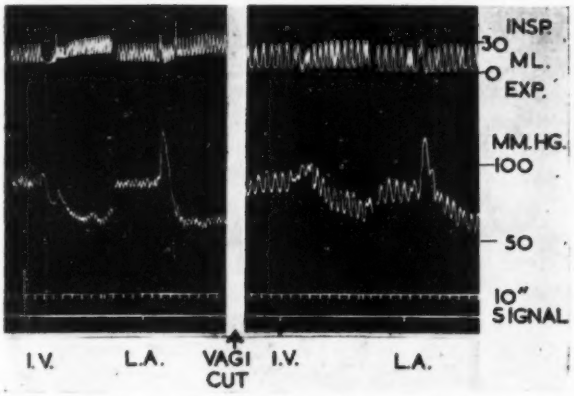

FIG. 1.—Cat 2 kg. Chloralose 60 mg./kg., atropine 2 mg. Upper record, respiration; lower record, carotid blood pressure. At the signal marks 90 μg. of 5-hydroxytryptamine was injected intravenously (I.V.) or into the left atrium (L.A.).

dose injected intravenously had previously caused a transient cessation of respiratory movements. In two of the six cats the apnoea seen on intravenous injection of 5-hydroxytryptamine was not abolished by vagotomy.

Effect of Vagal Cooling on the Reflex Apnoea.—The effect of vagal cooling on the apnoea caused by intravenous injection of 5-hydroxytryptamine was examined in three cats; in two of them vagotomy did not completely abolish the apnoea caused by the intravenous injection of 5-hydroxytryptamine, and the results were therefore equivocal. In a third cat the apnoea caused by the intravenous injection of 45 μg. 5-hydroxytryptamine survived cooling of the vagi to 2.5° C., but was abolished by vagotomy (Fig. 2). This figure also shows that the apnoea caused by an intravenous injection of 100 μg. of 2-α-napthyl ethyl isothiourea (another amidine compound whose actions in the cat resemble closely those of phenyl diguanide) likewise survived cooling of the vagi to 2.5° C. and was also abolished by vagotomy.

Effect of 5-Hydroxytryptamine on Discharge of Afferent Vagal Fibres.—There was no significant change of activity in four of five slowly adapting pulmonary stretch fibres on which the effect of 5-hydroxytryptamine was tested. In one (Fig. 3) a continuous low frequency discharge occurred 9 sec. after injection of 50 μg. during the initial period of respiratory inhibition. On plotting frequency of impulses before and after injection of the drug against intrapleural pressure no evidence of sensitization was found; other factors appear to have been concerned. It seems, therefore, that 5-hydroxytryptamine, like phenyl diguanide, does not sensitize or stimulate the pulmonary stretch receptors.

The drug had no significant effect on one depressor fibre and one right atrial type B fibre (Paintal, 1953a). In one left atrial type B fibre there occurred a threefold increase in activity (Fig. 3) beginning 9 sec. after injection of the drug. This happened some time after bradycardia and respiratory inhibition had started, and was preceded by a decrease in activity. It could not be ascertained with certainty whether this was due to sensitization of the receptor, as there was no record of left intra-atrial pressure.

On three occasions in three different cats 5-hydroxytryptamine aroused activity in previously inactive fibres (Fig. 4). The character of the response was in all instances the same as the response of these fibres to 100 μg. phenyl diguanide (Paintal,

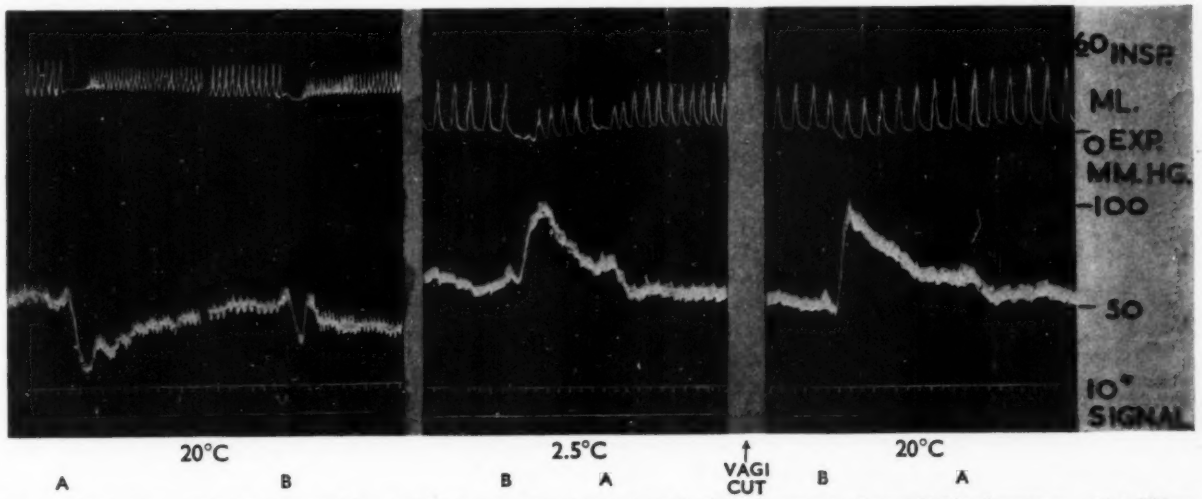

FIG. 2.—Cat 2.6 kg. Chloralose 60 mg./kg., atropine 3 mg. Upper record, respiration; lower record, carotid blood pressure. At A 100 μg. of 2-α-naphthyl ethyl isothiourea, and at B 45 μg. of 5-hydroxytryptamine, were injected. The first section is at 20° C., the second with the vagi cooled for 2 min. to 2.5° C., and the third after vagotomy at 20° C.

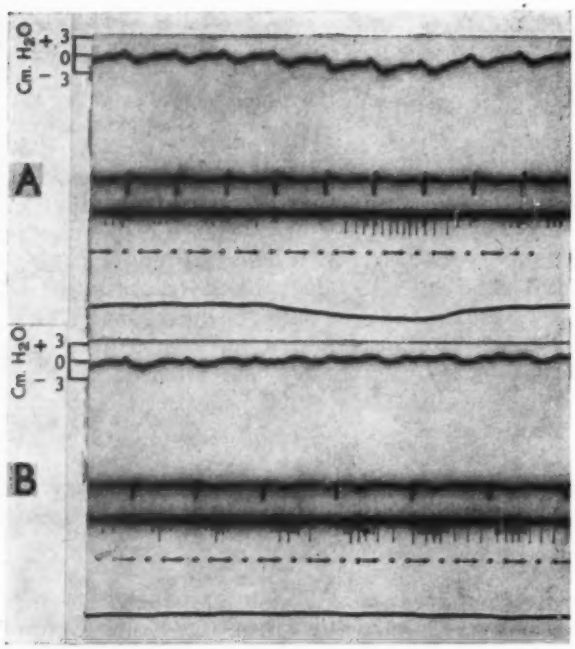

FIG. 3.—Cat, chloralose 80 mg./kg. Effect of 50 μg. 5-hydroxytryptamine on vagal afferent discharges; A before, B after intravenous injection. From above downwards, right venous pressure; E.C.G.; impulses in a vagal strip; time in 1/10 sec.; intrapleural pressure, inspiration downwards. The fibre with a small spike discharge is a left atrial fibre, the one with large spikes arises from a pulmonary stretch receptor. Note that the increase in atrial fibre activity in B follows the rise in right venous pressure.

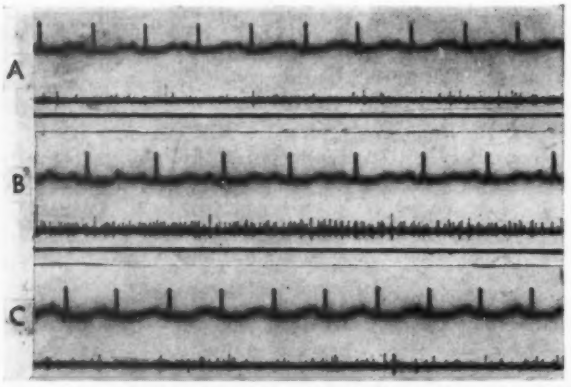

FIG. 4.—Cat, chloralose 80 mg./kg. Vagal afferent fibres stimulated by an intravenous injection of 25 μg. 5-hydroxytryptamine. Upper record, E.C.G.; lower record, activity in a vagal strip. A before, B 13 sec. after, and C 24 sec. after injection.

1953c). One single active unit was affected in the same way by both phenyl diguanide and 5-hydroxytryptamine. The response, as with phenyl diguanide, could be repeated several times, but the effect of 5-hydroxytryptamine differed from that of phenyl diguanide in that the degree of respiratory inhibition did not seem to be related to the frequency and number of units contributing to the discharge. Although the discharge resembled that evoked by phenyl diguanide in that it started 2 to 4 sec. after injection, it always lasted longer. On the other hand, respiratory inhibition was much more prolonged with phenyl diguanide than with 5-hydroxytryptamine, and with the latter the discharge always outlasted the respiratory inhibition by several seconds. The conduction velocity of one of these fibres was 5 m./sec. As with units stimulated by phenyl diguanide, none of these fibres was affected by gradual or rapid inflations of the lungs, suction of air from the trachea, administration of 100% nitrogen or a mixture of 90% oxygen + 10% carbon dioxide, or by intratracheal inhalation of ammonia. The effect of 5-hydroxytryptamine was only investigated in fibres stimulated by phenyl diguanide and such others as have been mentioned; the possibility therefore exists that the drug might excite fibres not affected by phenyl diguanide.

DISCUSSION

It is clear that 5-hydroxytryptamine can cause a reflex apnoea in cats by the stimulation of receptors situated in the circulation between the great veins and the left atrium. As this response is abolished by vagotomy, the afferent path must run in the vagi, but since the reflex survives vagal cooling to 2.5° C. it cannot be due to the stimulation of pulmonary stretch endings whose activity is abolished by cooling the vagi to 10–12° C. (Dawes, Mott, and Widdicombe, 1951). The respiratory reflex described in this paper could be due to the stimulation of those pulmonary receptors responding to various amidine compounds which also cause a reflex apnoea in expiration, abolished by vagotomy but not by cooling the vagi to 2.5° C. and unaffected by thoracic sympathectomy (Dawes, Mott, and Widdicombe, 1951). Further weight is added to this hypothesis by the observation that 5-hydroxytryptamine excites those vagal fibres which are otherwise excited only by phenyl diguanide. The analysis of the respiratory effects of 5-hydroxytryptamine is complicated by the fact that doses sufficient to cause the reflex apnoea may also cause an apnoea, possibly of central origin, and not abolished by vagotomy; an examination of the records suggests that other respiratory phenomena may occur as well.

The fact that the fibre activity produced by 5-hydroxytryptamine is greater and more prolonged than that produced by phenyl diguanide, while the inhibition of respiratory movements produced by the former is much less, also suggests that the mechanisms concerned with 5-hydroxytryptamine are less simple than with phenyl diguanide. 5-Hydroxytryptamine has, among other effects, a

direct action on bronchial musculature and the pulmonary vessels (Gaddum and Swan, personal communication).

The variability of the results of our pharmacological experiments recalls the experience of Douglas and Toh (1952) with this compound in dogs. Page (1952) states that 5-hydroxytryptamine in cats causes an apnoea followed by hyperpnoea which is not influenced by vagotomy. This result is not necessarily inconsistent with the findings of this paper.

There therefore seems little doubt that the mechanism of the reflex apnoea caused by 5-hydroxytryptamine in cats is identical with that evoked by phenyl diguanide. There is no evidence that the slowly adapting pulmonary stretch receptors, or the endings of the depressor or right or left atrial fibres, are involved.

The normal function of the nerve endings stimulated by phenyl diguanide and 5-hydroxytryptamine is not known. 5-Hydroxytryptamine is known to stimulate pain receptors in man (Armstrong, Dry, Keele, and Markham, 1952), and, if it does so in the cat as well, then it is possible that the fibres sensitive to phenyl diguanide and 5-hydroxytryptamine might arise from pain receptors in the lungs. The evidence for the presence of such receptors in the lungs is small, but it is significant that pain is a prominent feature of bronchogenic carcinoma (Jackson, 1942). Such a hypothesis would explain the fact that these fibres are normally quiescent and also that they are unaffected by procedures that arouse activity in other respiratory and cardiovascular receptors. Further investigation is required to elucidate this point.

Summary

1. 5-Hydroxytryptamine injected intravenously into some cats in doses of 15–45 μg./kg. body weight causes a reflex apnoea which is abolished by vagotomy; in such cats this quantity of 5-hydroxytryptamine has little effect on respiration when injected into the left atrium. In other cats this compound causes an apnoea which is not abolished by vagotomy.

2. The reflex apnoea caused by 5-hydroxytryptamine resembles that described for various amidine compounds in the following features: (*a*) it arises from the vessels between the great veins and the left atrium; (*b*) it is abolished by cutting the vagi but not by cooling them to 2°–3° C.

3. Afferent vagal fibres which are stimulated by phenyl diguanide are also stimulated by 5-hydroxytryptamine. These fibres have a conduction velocity of 5–6 m./sec.

4. There is no evidence that 5-hydroxytryptamine stimulates or sensitizes pulmonary stretch, right or left atrial, or depressor nerve endings.

We wish to thank Professor J. H. Gaddum, F.R.S., for a supply of 5-hydroxytryptamine creatinine sulphate.

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THE EFFECT OF DESOXYCORTICOSTERONE ACETATE ON THE RELEASE OF ADRENOCORTICOTROPHIN BY THE PITUITARY GLAND

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The results of experiments performed in many laboratories indicate that the adrenocorticotrophic activity of the pituitary gland is controlled, to some extent, by the level of adrenocortical hormones in the blood. Many workers have shown that the chronic administration of adrenocortical extracts to rats results in atrophy of the adrenal cortex. Sayers and Sayers (1947) and Long (1947) demonstrated, in rats, that the fall in adrenal ascorbic acid concentration, which normally follows the application of various types of stress stimuli, could be prevented by previous treatment of the animals with adrenocortical hormones.

Sayers and Sayers (1947) found that several crystalline cortical steroids, including desoxycorticosterone, were effective in inhibiting pituitary adrenocorticotrophic activity. However, Moya and Selye (1948), Hall, Finerty, Hall, and Hess (1951), and Gershberg, Fry, Brobeck, and Long (1950) did not find that DOCA prevents the release of ACTH in response to stress.

The experiments which form the subject of this paper were carried out to re-examine the effect of DOCA on the stress-induced secretion of ACTH and to determine whether this steroid possesses any pituitary inhibitory effect. Changes in the concentration of ascorbic acid in the adrenal glands were used as indices of increased adrenocortical, and hence of adrenocorticotrophic, activity. The form of stress employed was unilateral adrenalectomy under ether anaesthesia.

MATERIALS AND METHODS

The experiments were performed on male albino Wistar rats, weighing from 130 to 250 g. and maintained on a diet of cubes, cabbage, and water.

Twenty-four hours before being used the rats were transferred to a room in which the temperature was thermostatically controlled at 70° F. and where the experiments were performed. During the course of the experiments the animals were given access to food and water.

DOCA.—Deoxycortone acetate, *B.P.* (Organon), was used. Stock solutions were prepared containing 5, 10, 15, and 20 mg. DOCA per ml. in arachis oil. The DOCA tended to crystallize out from the solutions of highest concentration, when the solutions were allowed to stand, but the steroid redissolved readily on heating. The injections of DOCA were administered in volumes of 1 ml. per 100 g. body weight of the animals. The solutions were warmed to 37–40° C. before injection to facilitate administration and to keep the highest concentration of DOCA in solution.

Removal and Analysis of Adrenal Glands

The removal of adrenal glands from dead or anaesthetized animals was carried out by the dorsal approach. Excised adrenal glands were dissected free from fat and connective tissue with a fine pair of scissors. Care was taken to remove all traces of extra-adrenal tissue without damaging the capsule. The glands were weighed as quickly as possible to minimize the loss of moisture, and placed in test-tubes containing a little acid-washed sand and 4% trichloracetic acid solution. They were minced with the aid of a glass rod and the sand, and the tubes were stored in a refrigerator overnight. The ascorbic acid contents of the tubes were determined on the following day by the method of Roe and Kuether (1943). The results were expressed as mg. ascorbic acid per 100 g. adrenal tissue.

RESULTS

The Effect of Unilateral Adrenalectomy on the Ascorbic Acid Concentration in the Intact Adrenal Glands of Normal Rats

One hundred and eight animals were used in this experiment. Control animals were killed by a blow on the head and their adrenal glands were removed as quickly as possible. Test animals were anaesthetized with ether and their left adrenal glands were removed and discarded. The skin incisions were closed and the animals were allowed to recover from the operations. The rats were killed after various time intervals after unilateral adrenalectomy. The right adrenals were removed,

TABLE I

ASCORBIC ACID CONCENTRATIONS IN THE REMAINING ADRENAL GLANDS OF RATS AT VARIOUS TIME INTERVALS AFTER UNILATERAL ADRENALECTOMY

Controls A: adrenals removed from freshly killed rats.
Controls B: adrenals removed immediately after ether anaesthesia.

No. of Rats	Time Interval in Hours Between Unilateral Adrenalectomy and Sacrifice	Mean Ascorbic Acid Concentration mg./100 g. Adrenal Tissue ± Standard Error
24	Controls A	438±5
12	Controls B	367±8
12	½	290±15
12	1	264±12
12	2	251±8
12	4	261±16
12	8	363±18
12	16	475±17

and their ascorbic acid contents were determined. The members of one group of animals were anaesthetized with ether and their right adrenal glands, removed under anaesthesia, were assayed for ascorbic contents.

The results obtained are shown in Table I and Fig. 1.

The values obtained for the concentration of ascorbic acid in the adrenal glands of control animals were very constant. In general, the left adrenal gland of the male albino rat was found to be heavier than the right and contained more ascorbic acid, but, when the results were expressed as concentrations of ascorbic acid per 100 g. adrenal tissue, there was good agreement between the values for the right and left glands in the same animal.

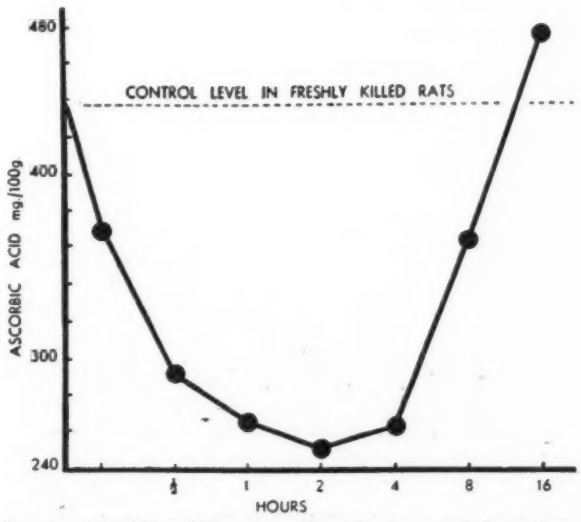

FIG. 1.—Ascorbic acid concentrations in the remaining adrenal glands of rats at various time intervals after unilateral adrenalectomy. Ordinate: Adrenal ascorbic acid concentrations. Abscissa: Time interval between unilateral adrenalectomy and sacrifice.

Unilateral adrenalectomy under ether anaesthesia resulted in a very rapid depletion of the ascorbic acid concentration in the intact adrenal glands. The concentration of adrenal ascorbic acid fell quickly and reached its minimum value of approximately 58% of the normal level, one to two hours after the performance of the operation. This low adrenal ascorbic acid concentration was maintained for four hours. After eight hours the level of ascorbic acid had commenced to return to normal. After sixteen hours the mean concentration was slightly higher than normal.

The time taken to anaesthetize a rat and to remove one of its adrenal glands was approximately four minutes. The fall in adrenal ascorbic acid concentration was found to take place very rapidly. Four minutes from the commencement of anaesthesia there had occurred a significant ($P<0.01$) fall in adrenal ascorbic acid concentration to approximately 84% of the control level. It was for this reason that in the next experiment the adrenal glands first removed from the animals were discarded, and normal unoperated rats were used as controls.

The Effect of Unilateral Adrenalectomy on the Ascorbic Acid Concentration in the Intact Adrenal Glands of Rats Pre-treated with Desoxycorticosterone Acetate

The experiment was carried out on 600 rats. The animals were divided into two main groups. The members of the first group received intraperitoneal or subcutaneous injections of various doses of DOCA, and at specific time intervals afterwards half the number in the group were anaesthetized with ether and their left adrenal glands were removed and discarded. One hour later these unilaterally adrenalectomized animals were killed. The right adrenal glands were removed and transferred to trichloracetic acid solution for the determination of their ascorbic acid contents. The unoperated animals in the group were killed at the same time after receiving the injections of DOCA. Their right adrenals were removed and estimated for ascorbic acid contents. The rats in the second group received injections of arachis oil instead of DOCA. Subsequently they were subjected to the same treatment as the members of the first group. A number of control animals were killed without any previous treatment.

Solutions of DOCA in arachis oil were injected intraperitoneally in doses of 5, 10, and 15 mg., and subcutaneously in doses of 5, 10, and 20 mg. DOCA per 100 g. body weight. Both the DOCA solutions and arachis oil were given in volumes of 1 ml. per 100 g. Preliminary experiments had

TABLE II

ASCORBIC ACID CONCENTRATIONS IN THE INTACT ADRENAL GLANDS OF RATS FOLLOWING UNILATERAL ADRENALECTOMY PERFORMED AFTER THE INJECTION OF DOCA

Test animals were unilaterally adrenalectomized at various time intervals (indicated in the Table) after the injection of DOCA, and the concentrations of ascorbic acid in their intact adrenals were determined one hour later

Control rats were killed without previous surgical interference, and their adrenal ascorbic acid concentrations were determined. The figures in brackets in the last column refer to the number of rats in the group. — = controls killed; i.p. = intraperitoneally; s.c. = subcutaneously

Dose of DOCA mg./100 g. Body Weight	Time Interval in Hours Between Injection and Operation	Mean Ascorbic Acid Concentrations mg./100 g. Adrenal Tissue ± Standard Error
0 i.p.	—	444±17 (6)
0 ,,	1	273±25 (6)
5 ,,	—	442±31 (6)
5 ,,	1	357±21 (6)
10 ,,	—	415±13 (6)
10 ,,	1	374±34 (6)
15 ,,	—	355±15 (6)
15 ,,	1	374±25 (6)
0 s.c.	—	373±34 (6)
0 ,,	1	259± 9 (6)
5 ,,	—	448± 8 (8)
5 ,,	1	279±10 (8)
10 ,,	—	404±16 (8)
10 ,,	1	271± 8 (8)
20 ,,	—	391±13 (8)
20 ,,	1	258±16 (8)
0 i.p.	—	419± 7 (6)
0 ,,	2	216± 9 (6)
5 ,,	—	398±18 (6)
5 ,,	2	351±33 (6)
10 ,,	—	432±18 (6)
10 ,,	2	354±21 (10)
15 ,,	—	387±23 (6)
15 ,,	2	365±27 (6)
0 s.c.	—	368±15 (6)
0 ,,	2	239±11 (6)
5 ,,	—	401±10 (8)
5 ,,	2	253±16 (8)
10 ,,	—	357±12 (8)
10 ,,	2	266± 9 (8)
20 ,,	—	383±15 (8)
20 ,,	2	273±16 (8)
0 i.p.	—	457± 8 (6)
0 ,,	4	269±15 (6)
5 ,,	—	438±11 (6)
5 ,,	4	315±16 (6)
10 ,,	—	432±15 (6)
10 ,,	4	409±24 (10)
15 ,,	—	424±16 (6)
15 ,,	4	433±18 (6)
0 s.c.	—	396±10 (6)
0 ,,	4	274±11 (6)
5 ,,	—	404±10 (8)
5 ,,	4	375±25 (8)
10 ,,	—	423±19 (8)
10 ,,	4	382±12 (8)
20 ,,	—	407±17 (8)
20 ,,	4	361±12 (8)
0 i.p.	—	421±10 (6)
0 ,,	8	264±10 (6)
5 ,,	—	—
5 ,,	8	262±11 (6)
10 ,,	—	—
10 ,,	8	284±21 (6)
15 ,,	—	425±16 (6)
15 ,,	8	360±15 (6)
0 s.c.	—	429±25 (6)
0 ,,	8	275±14 (6)
5 ,,	—	429± 8 (8)
5 ,,	8	323±13 (8)
10 ,,	—	424±10 (8)
10 ,,	8	373±24 (8)
20 ,,	—	424±10 (6)
20 ,,	8	378±30 (6)
0 i.p.	—	453±21 (6)
0 ,,	16	260±11 (6)
5 ,,	—	—

TABLE II—*continued*

Dose of DOCA mg./100 g. Body Weight	Time Interval in Hours Between Injection Between Operation	Mean Ascorbic Acid Concentrations mg./100 g. Adrenal Tissue ± Standard Error
5 i.p.	16	277±16 (6)
10 ,,	—	—
10 ,,	16	287±17 (6)
15 ,,	—	430±16 (6)
15 ,,	16	284±20 (6)
0 s.c.	—	460±15 (6)
0 ,,	16	288± 5 (6)
5 ,,	—	429±19 (8)
5 ,,	16	307±14 (8)
10 ,,	—	420±10 (8)
10 ,,	16	402± 9 (8)
20 ,,	—	475±10 (6)
20 ,,	16	453±20 (6)
0 s.c.	—	516± 7 (6)
0 ,,	32	273±13 (6)
5 ,,	—	462±20 (6)
5 ,,	32	315±26 (6)
10 ,,	—	468±16 (6)
10 ,,	32	384±40 (6)
20 ,,	—	479±16 (6)
20 ,,	32	475±12 (6)

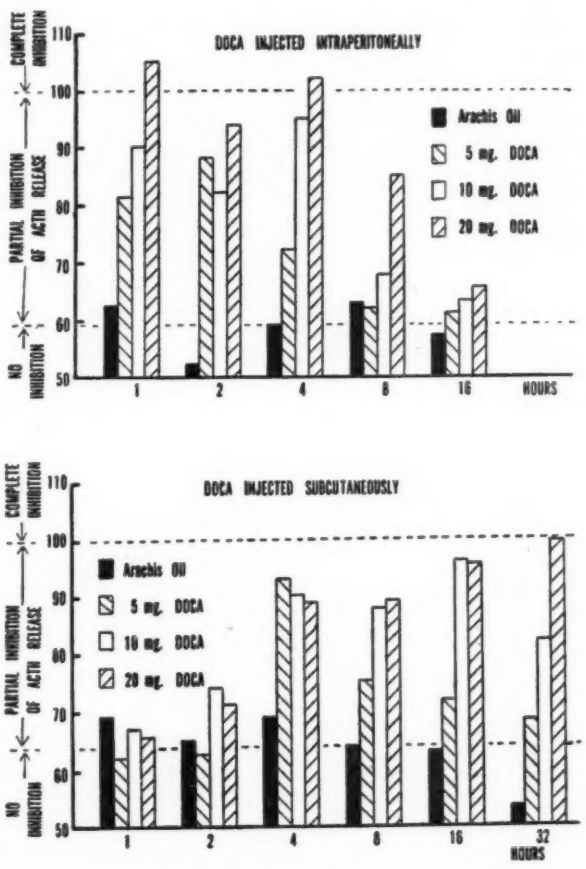

FIG. 2.—The effect of DOCA on the adrenal ascorbic acid depletion caused by unilateral adrenalectomy. Ordinate: Adrenal ascorbic acid concentration, one hour after the operation, expressed as a percentage of the control level in unoperated rats. The figures below the columns represent the time interval in hours between the injections and the performance of unilateral adrenalectomy.

shown that DOCA administered intraperitoneally in doses of 20 mg. or more per 100 g. constituted a severe form of stress and resulted in prolonged adrenal ascorbic acid depletion. For this reason, 15 mg. per 100 g. was the largest dose of DOCA administered by the intraperitoneal route. The results obtained are summarized in Table II and Fig. 2.

Intraperitoneal injections of arachis oil in the volumes used and of DOCA in doses equivalent to 5 and 10 mg. per 100 g. body weight produced no marked changes in adrenal ascorbic acid concentration. The administration of 15 mg. DOCA by the intraperitoneal route produced a definite fall in the concentration of ascorbic acid in the adrenal glands, but the levels had returned to normal four hours after the DOCA had been injected. The subcutaneous injection of arachis oil and the DOCA solutions caused no marked adrenal ascorbic acid depletion.

Unilateral adrenalectomy under ether anaesthesia produced an adrenal ascorbic acid depletion in rats which had been pre-treated with arachis oil only similar to that observed in the previous experiment on normal rats (black columns of Fig. 2). Pre-treatment of the rats with desoxycorticosterone acetate reduced the changes in adrenal ascorbic acid concentration which normally followed unilateral adrenalectomy.

The intraperitoneal administration of 5 mg. DOCA partially prevented the fall in adrenal ascorbic acid which resulted from the stress of the operation for a period of one to four hours after the DOCA had been injected. The 5 mg. dose of desoxycorticosterone acetate, administered subcutaneously, partially prevented the adrenal ascorbic acid depletion, caused by the operation, four to 32 hours after it had been injected.

Partial prevention of the fall in the concentration of ascorbic acid by 10 mg. DOCA was most marked four hours after it had been injected intraperitoneally and 16 hours after its subcutaneous administration.

Fifteen mg. DOCA, given intraperitoneally, completely prevented the adrenal ascorbic acid depletion due to unilateral adrenalectomy one, two, and four hours after its injection. This dose itself produced a fall in adrenal ascorbic acid concentration, and therefore its effect in preventing adrenal ascorbic acid depletion was most obvious four hours after it had been injected, when the control adrenal ascorbic acid level had returned to normal.

Twenty mg. DOCA, injected subcutaneously, completely prevented any stress-induced change in adrenal ascorbic acid concentration 16 and 32 hours after its administration.

About one-third of the total number of the rats which received the greatest doses of DOCA by the intraperitoneal route collapsed after the administration of the steroid. The animals passed into a coma half to one hour after the DOCA had been injected. In the comatose animals, respiration was hardly detectable, the heart rate was slow, and the skin of the feet and face was cyanosed. Only a few of the comatose rats showed no reflex responses to pinching the feet or tail. The majority still reacted to these stimuli, and showed exaggerated responses to the sound stimulus caused by opening and shutting their cage doors.

Subsequent experiments showed that, if these " anaesthetized " rats were allowed to recover, they regained consciousness six to eight hours later and resumed apparently normal activity. It was found that there existed no correlation between the animals which exhibited the most obvious symptoms of DOCA intoxication and those which showed most marked adrenal ascorbic acid depletion. None of the rats which were given subcutaneous injections of DOCA exhibited this phenomenon. The " anaesthetic " effect of large doses of steroids was first noticed by Selye (1941). More recently Cosmos, Duell, and Gaunt (1950) have studied similar effects produced by the administration of desoxycorticosterone glycoside.

Discussion

There is little doubt that the secretion of ACTH is partly controlled by a peripheral humoral mechanism, i.e., by the level of cortical hormones in the blood. However, there is much confusion in the literature concerning the ability of desoxycorticosterone acetate to depress the increased secretion of ACTH which normally follows stress.

On the one hand, a considerable amount of evidence exists that DOCA can block effectively the output of ACTH. Selye and Dosne (1942) found that the previous administration of DOCA to rats partially prevented the increase in adrenal weight caused by stress. Woodbury, Cheng, Sayers, and Goodman (1950) found, in rats, that the adrenal atrophy caused by DOCA could be prevented by the simultaneous administration of ACTH. They considered that some of the toxic effects of DOCA might be due to the inhibition of pituitary adrenocorticotrophic activity, resulting in adrenocortical insufficiency. Cheng and Sayers (1949) showed that large doses of DOCA, implanted as pellets, caused increased sensitivity to insulin in adrenal-demedullated rats. They attri-

buted this hypersensitivity to adrenocortical insufficiency caused by the inhibition of the adrenocorticotrophic activity of the pituitary gland. Sayers and Sayers (1947) reported that the adrenal ascorbic acid depletion caused by cold (4° C. for one hour) could be prevented by previous treatment of the animals with only 200 μg. desoxycorticosterone (per 100 g. body weight), injected subcutaneously in oil, immediately before the application of the stress.

On the other hand, many workers have been unable to demonstrate the pituitary inhibitory effect of DOCA. Moya and Selye (1948) found that "anaesthetic" doses of DOCA (12–14 mg. per 80–100 g. rat), administered intraperitoneally in vegetable oil, failed to prevent the fall in adrenal ascorbic acid concentration caused by exposure of the rats to cold. They suggested that, in severe stress, the secretion of adrenocorticotrophic hormone is controlled by a mechanism which the "peripheral-humoral" theory fails to explain. Hall, Finerty, Hall, and Hess (1951) found that desoxycorticosterone acetate administered acutely (24 mg. per 80–100 g. rat intraperitoneally in vegetable oil) or chronically (50 mg. per 80–100 g. rat subcutaneously as pellets) caused no adrenal ascorbic acid depletion. They reported that these large doses of DOCA failed to prevent the fall in adrenal ascorbic acid concentration caused by the stress of scalding. Gershberg *et al.* (1950) found that desoxycorticosterone acetate (4 mg. per 100 g. body weight), administered subcutaneously as an oily solution or an aqueous suspension, was ineffective in preventing the adrenal ascorbic acid depletion caused by the exposure of rats to cold.

The present work showed that previous treatment of rats with large doses of desoxycorticosterone acetate could prevent the fall in ascorbic acid concentration which occurs in the intact adrenal glands after unilateral adrenalectomy. The effect depended upon the dose of DOCA administered, its route of administration, and the time interval between the injection of the steroid and the application of the stress. In these experiments the degree of inhibition of pituitary adrenocorticotrophic activity produced by the DOCA was taken to be inversely proportional to the adrenal ascorbic acid depletion which occurred one hour after unilateral adrenalectomy. Where complete pituitary inhibition was obtained, ascorbic acid levels in the intact adrenal glands were not affected by the anaesthetic and the operation, and were equal to the concentrations found in the adrenals of DOCA-injected, unoperated, control animals. Where no inhibition of adrenocorticotrophic activity occurred the removal of one adrenal gland resulted in the usual considerable fall of ascorbic acid concentration in the other. It was considered that partial pituitary inhibition had been produced when intermediary adrenal ascorbic acid levels were obtained.

These experiments confirmed the observations of Sayers and Sayers (1947) that DOCA can cause inhibition of the adrenocorticotrophic activity of the pituitary gland. However, it was found that the doses of DOCA required to prevent the increased secretion of ACTH, in response to the stress of ether anaesthesia and unilateral adrenalectomy, were fifty to one hundred times as great as those which Sayers and Sayers (1947) found necessary to prevent the effect of cold. The results are in complete contrast to the findings of Moya and Selye (1948), Hall *et al.* (1951), and Gershberg *et al.* (1950). It is probable that Gershberg *et al.* (1950) were unsuccessful in demonstrating the pituitary inhibitory effect of DOCA, since they subjected their rats to stress too soon after the subcutaneous administration of the steroid. It is more difficult to explain the discrepancies which exist between the results obtained in the present work and those of Hall *et al.* (1951) and Moya and Selye (1948).

It is doubtful whether these results augment the evidence that the rate of secretion of ACTH by the adenohypophysis is controlled by a peripheral-humoral mechanism. They have shown that pituitary adrenocorticotrophic activity can be prevented by the previous treatment of rats with DOCA, but the required doses of the steroid were necessarily so great that the physiological significance of the results is obscure. Sayers and Sayers (1947) were successful in obtaining inhibition of pituitary adrenocorticotrophic activity with small doses of cortical steroids, and their results are more in accord with the existence of a peripheral-humoral mechanism for the control of the secretion of ACTH.

One reason for carrying out these experiments was to examine the possibility of modifying the Sayers' adrenal ascorbic acid depletion technique for the bioassay of ACTH (Sayers, Sayers, and Woodbury, 1948) by substituting rats, in which pituitary adrenocorticotrophic activity had been "blocked" with DOCA, for hypophysectomized animals. It was found that injected ACTH still caused adrenal ascorbic acid depletion in rats pretreated with DOCA. Preliminary attempts to make this finding the basis of a bioassay method for ACTH were described recently by Buttle and Hodges (1952) and Reiss, Halkerston, Badrick, and Halkerston (1951).

Since ACTH was still effective in producing adrenal ascorbic acid depletion, it was considered that DOCA had not prevented the stress-induced fall in adrenal ascorbic acid concentration by a direct action on the adrenal cortex. Similar evidence that adrenocortical hormones inhibit pituitary adrenocorticotrophic activity directly, rather than by interfering with the release of steroids from the adrenal cortex, was obtained by Ingle and Kendall (1937) and Sayers and Sayers (1947).

Summary

1. Unilateral adrenalectomy under ether anaesthesia caused marked ascorbic acid depletion in the intact adrenal glands of normal rats. Maximal effects were produced one to two hours after the performance of the operation.

2. Intraperitoneal injections of desoxycorticosterone acetate in arachis oil caused no fall in the adrenal ascorbic acid concentration in rats, when the doses of DOCA employed were not greater than 10 mg. per 100 g. body weight. Larger doses of DOCA administered by the intraperitoneal route caused adrenal ascorbic acid depletion. Subcutaneous injections of DOCA in arachis oil in doses of up to 20 mg. per 100 g. caused no fall in adrenal ascorbic acid concentration.

3. Pre-treatment of rats with large doses of DOCA tended to prevent the fall in adrenal ascorbic acid caused by unilateral adrenalectomy. The adrenal ascorbic acid depletion, which normally followed the operation, was completely prevented by (*a*) the intraperitoneal administration, one to four hours previously, of 15 mg. DOCA, and (*b*) the subcutaneous administration, 16 to 32 hours previously, of 20 mg. DOCA per 100 g. body weight.

My most sincere thanks are due to Professor G. A. H. Buttle for his constant advice and helpful criticism of the work. I am grateful to Miss D. Bathgate and Mr. G. S. Cox for invaluable technical assistance. Part of the work was done during the tenure of a Wellcome Pharmaceutical Research Fellowship.

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EFFECT OF SYMPATHETIC DENERVATION ON THE CHOLINESTERASE IN THE NICTITATING MEMBRANE AND THE IRIS

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The work to be described in this paper is a continuation of observations already made in this laboratory on the effect of nerve degeneration on the enzymes in various tissues. Robinson (1952) found that amine oxidase was present in the nictitating membrane and in the iris of the cat, and Burn and Robinson (1952) studied the effect of removing the superior cervical ganglion on the amount present. In the nictitating membrane they found that the degeneration of the sympathetic nerves caused a fall which reached a minimum about the 10th–12th day after extirpation of the ganglion. The amount of amine oxidase then rose and returned to normal by the 35th day. The restoration of the enzyme was difficult to explain except by supposing that regeneration of nerve fibres in the nictitating membrane had occurred, and in a further investigation (Burn and Robinson, 1953) it was found that when the stellate ganglion was removed as well as the superior cervical ganglion the fall in amine oxidase was greater and there was no recovery. In addition to the observations on the nictitating membrane which were made in large numbers of cats, a few observations were made on the effect of degeneration of the sympathetic fibres on the amine oxidase in the iris and in the arteries of the cat's foreleg. These indicated that a fall occurred in the iris and in the blood vessels also, but they require confirmation on account of their small number, especially those in the blood vessels in which only three manometric experiments were performed.

An investigation was also undertaken by Schofield (1952) on the cholinesterase present in the iris of the cat. It is innervated by fibres coming from the ciliary ganglion. After extirpation of the ganglion it was found that there was a fall in cholinesterase to about 40% of the amount present in the corresponding normal iris, when acetyl-β-methylcholine was used as a substrate, and a fall to about 80% when benzoylcholine was used as a substrate.

These observations are of interest because they contribute to an explanation of the increased sensitivity of denervated structures. Thus the fall in amine oxidase in the nictitating membrane was found to be correlated with the increased sensitivity of the membrane to noradrenaline in a series of 25 cats. However, the fall in amine oxidase did not explain all the changes in the denervated membrane, since this becomes hypersensitive not only to noradrenaline and adrenaline but also to acetylcholine (Rosenblueth, 1932). In 1935 Bacq and Fredericq described experiments which indicated that some of the fibres in the sympathetic pathway to the nictitating membrane were cholinergic ; they found that stimulation of the cervical sympathetic had a greater effect when eserine was instilled into the conjunctival sac, and that it had a smaller effect after the administration of atropine. This result suggested to us that cholinesterase might be present in the nictitating membrane, and that the hypersensitivity to acetylcholine observed after degeneration of the sympathetic fibres might be due to a fall in its amount. The present experiments were therefore undertaken to see if cholinesterase was present in the nictitating membrane and to discover if the amount was modified by degeneration of the sympathetic nerves.

A further question arose out of the observations which had been thus far made. Experiments had shown that removal of the sympathetic fibres from the nictitating membrane led to a fall in amine oxidase, and that removal of the parasympathetic fibres from the iris led to a fall in cholinesterase. Would removal of sympathetic fibres from the iris affect cholinesterase? The experiments which were planned to investigate cholinesterase in the nictitating membrane afforded an opportunity of answering this question as well. Observations

were therefore made in the iris as well as in the nictitating membrane.

METHODS

Manometric Observations.—The nictitating membrane, after removal from the eyeball, was pinned out and dissected free from striated muscle, fat, the Harderian gland, and cartilage. The remaining tissue was placed in a mortar, chopped as finely as possible with scissors, frozen to −10° C. with 2 ml. bicarbonate Ringer solution, ground up until it thawed, and made up to 6.5 ml. with bicarbonate Ringer.

The iris, after cutting away the cornea, was gently lifted out with forceps. It was chopped and ground as described for the nictitating membrane and suspended in 3 ml. bicarbonate Ringer.

Cholinesterase was estimated by the manometric method, using acetylcholine, acetyl-β-methylcholine, and butyrylcholine as substrates, the final concentrations being 0.02M, 0.03M, and 0.02M respectively. The following quantities were used: substrate 0.3 ml., nictitating membrane suspension 1.5 ml., iris suspension 0.5 ml. with acetylcholine and acetyl-β-methylcholine as substrates, 1 ml. with butyrylcholine as substrate. The volume was brought to 3 ml. with bicarbonate Ringer. The gas mixture was nitrogen with 5% carbon dioxide. The course of the reaction was followed for 30 min. at 37.5° C.

Removal of the Ganglion.—A series of 15 cats were prepared by removal of the right superior cervical ganglion under ether anaesthesia using full aseptic precautions. Between 6–24 days later the cats were killed by coal gas and then bled from the carotid arteries.

RESULTS

Nictitating Membrane.—Cholinesterase was found to be present in the nictitating membrane, and its amount was estimated by the use of all three substrates. Figures for the right and left membranes of normal eyes were obtained for 6 cats in order to ensure that the method of estimation was satisfactory. On account of the difficulty of distinguishing between smooth muscle and the surrounding connective tissue in dissecting the membranes, the results were expressed, not per gramme of tissue, but per nictitating membrane. The results are shown in Table I, and the mean figures for the right and left eyes agreed closely for each of the three substrates. Thus when a group of 6 cats was studied reasonable uniformity was observed in the two eyes.

TABLE I

CHOLINESTERASE IN NORMAL NICTITATING MEMBRANES

μl. CO_2 per nictitating membrane per hr.

Acetylcholine		Acetyl-β-methylcholine		Butyrylcholine	
Right	Left	Right	Left	Right	Left
181	212	86	78	379	340
515	591	174	141	1,130	815
355	276	155	171	620	532
875	1,075	168	141	1,350	1,730
285	216	82	39	393	433
428	375	134	142	507	609
Mean 440	457	133	119	730	743

Results were then obtained in 14 cats in which the right superior cervical ganglion had been removed previously in a sterile operation. They are shown in Table II.

TABLE II

CHOLINESTERASE IN DENERVATED NICTITATING MEMBRANES

μl. CO_2 per nictitating membrane per hr.

Days Since Operation	Acetylcholine		Acetyl-β-methylcholine		Butyrylcholine	
	Denervated Right	Normal Left	Denervated Right	Normal Left	Denervated Right	Normal Left
6	367	698	90	163	723	1,210
8	647	560	125	169	1,650	1,180
8	227	261	0	45	710	796
9	311	193	100	88	555	536
10	394	397	60	54	1,015	872
11	286	391	81	91	679	857
13	662	645	98	117	1,368	1,095
13	379	388	56	87	858	955
14	247	450	17	65	804	933
14	587	702	155	291	1,010	890
14	449	289	208	161	774	863
19	480	352	103	140	1,110	1,130
21	266	167	0	37	805	725
24	825	722	242	220	980	1,390
Mean	445	444	95	123	931	959

The mean figures for the normal left membranes in Table II were close to the mean figures in Table I for the corresponding substrates except for butyrylcholine, for which the mean figures in Table II were higher. However, the results show that, while denervation did not modify the figures for acetylcholine and for butyrylcholine, it reduced the figures for acetyl-β-methylcholine. The mean difference between the 14 pairs of membranes when acetyl-β-methylcholine was the substrate was 28.1, and the standard error was 11.97. Hence $t=2.35$ and $P=0.037$.

Thus degeneration of the sympathetic fibres to the nictitating membrane caused a fall in true cholinesterase, but not in pseudocholinesterase.

Iris.—Estimates of the cholinesterase present in the normal irises of 8 cats are given in Table III. With each substrate the figures for the right eye were rather lower than the figures for the left eye.

The results of examining 15 cats in which the right iris was denervated by removal of the superior cervical ganglion are shown in Table IV.

TABLE III
CHOLINESTERASE IN THE IRIS
μl. CO_2/g./hr.

Acetylcholine		Acetyl-β-methylcholine		Butyrylcholine	
Normal Right	Normal Left	Normal Right	Normal Left	Normal Right	Normal Left
5,670	3,350	3,150	4,240	—	—
10,500	13,000	6,040	6,560	985	1,720
5,500	8,200	2,030	2,620	1,580	1,565
7,950	11,800	6,480	8,430	3,750	4,320
7,860	8,075	5,700	5,160	1,715	1,550
5,875	7,530	3,715	4,760	3,120	3,750
9,480	8,430	6,920	6,940	1,030	988
6,360	6,830	3,940	4,360	652	586
Mean 7,400	8,402	4,747	5,383	1,833	2,068

TABLE IV
CHOLINESTERASE IN THE IRIS
μl. CO_2/g./hr.

Days Denervated	Acetylcholine		Acetyl-β-methylcholine		Butyrylcholine	
	Denervated Right	Normal Left	Denervated Right	Normal Left	Denervated Right	Normal Left
6	4,180	4,420	2,840	3,400	881	1,438
8	5,520	6,080	3,290	4,280	2,510	3,325
8	6,920	11,900	4,595	7,500	1,360	3,490
9	8,980	8,525	5,170	3,960	1,050	1,355
10	8,350	9,725	3,265	7,250	1,700	2,175
11	3,030	6,095	1,795	3,790	1,230	925
12	5,330	6,750	3,570	4,670	—	—
13	8,100	10,150	5,480	6,840	1,550	2,480
13	7,670	8,090	4,730	6,550	2,380	3,420
14	4,930	6,715	2,510	2,220	1,190	1,550
14	2,840	9,400	1,940	7,090	873	1,970
14	8,060	8,700	5,780	6,100	1,430	1,625
19	6,405	5,730	4,050	4,170	1,340	2,770
21	6,420	6,430	5,640	5,360	220	1,322
24	8,530	8,440	5,590	6,430	3,080	4,805
Mean	6,351	7,810	4,016	5,370	1,485	2,332

Again the mean values for the right eye were found to be lower than those for the left eye with all three substrates, but whereas the difference was not much greater than the difference for normal right and normal left eyes with acetylcholine and acetyl-β-methylcholine, it was considerably greater with butyrylcholine, indicating that there was a fall in pseudocholinesterase as a result of denervation. Because of the difference in the results for normal right and normal left irises, the figure for each right iris was calculated as a percentage of the figure for the corresponding left iris. The mean results are given in Table V. They show that the cholinesterase was less in the denervated right iris than in the normal right iris with all three substrates. The difference was, however, not significant either for acetylcholine or for acetyl-β-methylcholine, but it was significant for butyrylcholine. The percentage was 93.5 for the normal right iris and it was 66.8 for the denervated right iris. The standard errors, calculated from the formula

$$\sqrt{\frac{Sd^2}{n(n-1)}}$$

were 7.31 and 7.21 respectively. Hence, using the formula

$$t=\frac{m_1-m_2}{\sqrt{\varepsilon_1^2+\varepsilon_2^2}},$$

$t=2.61$ and $P=0.017$. The value of t was also calculated as described by Burn, Finney, and Goodwin (1950), p. 42, and found to be 2.33, from which $P=0.032$. Thus removal of the sympathetic fibres to the iris was found to cause a fall in the pseudocholinesterase which was present.

TABLE V
MEAN VALUES FOR CHOLINESTERASE IN RIGHT IRIS AS PERCENTAGE OF LEFT

	Acetylcholine	Acetyl-β-methylcholine	Butyrylcholine
Normal right ..	95·6 (8)	87·4 (8)	93·5 (7)
Denervated right	83·5 (15)	79·9 (15)	66·8 (14)

Number of observations in brackets.

DISCUSSION

The finding that degeneration of the sympathetic fibres to the nictitating membrane was followed by a fall in amine oxidase (Burn and Robinson, 1952, 1953) served to explain the hypersensitivity which developed to noradrenaline and adrenaline, but it did not account for the hypersensitivity to acetylcholine observed by Rosenblueth (1932). We have looked, therefore, for the presence of cholinesterase in the nictitating membrane and, having found it, have studied the effect of degeneration of the sympathetic fibres upon its amount. We have observed a fall in true cholinesterase without a change in pseudocholinesterase. It is probable that the hypersensitivity of the denervated membrane to acetylcholine is due to this fall.

The observations which were made upon the iris were unexpected. The work of Schofield (1952) showed that degeneration of the ciliary nerves caused a fall in true cholinesterase to about 40% of the normal amount, and a slight fall in pseudocholinesterase. Since degeneration of the sympathetic fibres caused a fall in amine oxidase (Burn and Robinson, 1952) it seemed improbable that it would affect the cholinesterase. It was therefore of great interest to observe that a fall in pseudocholinesterase occurred.

In the denervated nictitating membrane the evidence is strong that the hypersensitivity to noradrenaline and adrenaline is due to a fall in amine

oxidase. By inference the hypersensitivity of the iris to these amines is also due to the fall in amine oxidase which occurs; it is, however, not a large fall, and we now have evidence that there is also a fall in pseudocholinesterase. The question therefore arises whether the fall in pseudocholinesterase plays any part in causing the hypersensitivity of the iris. Now Bülbring and Burn (1942) showed that the constrictor action of adrenaline in the vessels of the dog's hindleg when perfused with blood was augmented by the presence of neostigmine in the blood. This suggested that the constrictor action of adrenaline depended on the amount of cholinesterase, and that if this was reduced by the presence of an inhibitor the constrictor action was then greater. It is therefore possible that the hypersensitivity of the iris to adrenaline after degeneration of the sympathetic supply is due either wholly or in part to the fall in pseudocholinesterase. The dilatation of the iris by cocaine is generally explained as due to a potentiation of the action of adrenaline by cocaine. Burn and Robinson (1952), in discussing the action of cocaine on the nictitating membrane, suggested that its effects were due to an inhibition of amine oxidase which was demonstrated to occur *in vitro* by Philpot (1940). We must now remember that Blaschko, Chou, and Wajda (1947) found that cocaine inhibited the hydrolysis of benzoylcholine by horse serum, and that this inhibition was greater than the inhibition of true cholinesterase. Thus the possibility arises that in dilating the iris cocaine acts by inhibiting pseudocholinesterase. We may then go further and point out that cocaine also causes blanching of the conjunctiva, which is also said to be due to potentiation of the effect of adrenaline. Can this also be due to an inhibition of pseudocholinesterase?

Summary

1. Cholinesterase is present in the nictitating membrane of the cat both in the "true" and in the "pseudo" forms.

2. Removal of the superior cervical ganglion results in a fall in the amount of "true" cholinesterase, and this fall may explain the hypersensitivity of the denervated nictitating membrane to acetylcholine.

3. Removal of the superior cervical ganglion results in a fall in the pseudocholinesterase present in the iris.

4. The question is raised whether the hypersensitivity of the iris to adrenaline after removal of the superior cervical ganglion is due to the fall in pseudocholinesterase.

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A COMPARISON OF THE BIOLOGICAL PROPERTIES OF CEPHALOSPORIN N AND PENICILLIN

BY

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Since cephalosporin N (abbreviated to "N" in this communication), which has been shown to be a new type of penicillin by Abraham, Newton, Crawford, Burton, and Hale (1953), differs strikingly from the previously known natural penicillins, it was decided to compare its behaviour towards bacteria and in the animal body with that of penicillin G.

MATERIALS AND METHODS

Materials.—We are indebted to the Antibiotics Research Station of the Medical Research Council for the preparation of the N. Nearly all the work was done with material having 25 units/mg., though for a few experiments samples having 29 and 32 units/mg. were taken. These arbitrary units are not in any way related to penicillin units, nor has the potency of N been expressed in terms of penicillin units or vice versa. It seems likely that pure N will have a potency of the order of 80 arbitrary units/mg.

For comparison, crystalline sodium penicillin G (Glaxo or Boots) of not less than 1,600 i.u./mg. was used. It was not weighed out, but the phials were assumed to contain the 100,000 or 200,000 units stated; inaccuracies which could thus result were partly eliminated by using as assay standard for any given experiment part of the solution actually used for that experiment.

Antibacterial Action.—Titrations were carried out in fluid media, using twofold dilutions throughout.

Assay of Antibiotics in Body Fluids.—In urine both antibiotics were assayed by the cylinder-plate method, using plates bulk-seeded with *Staph. aureus* NCTC 6571 for penicillin and with an avirulent *S. typhi* for N. Samples were diluted in phosphate buffer *p*H 6.8, and a standard curve was prepared for each set of assays from solutions of known strength made up in the same buffer.

For blood and cerebrospinal fluid assays, the cylinder-plate method required too much fluid and/or was insufficiently sensitive. Various micro-dilution methods with media containing fermentable sugars and an indicator (Florey *et al.*, 1949) were only moderately satisfactory, since the end-point was not sharp in the presence of serum. A micro vertical diffusion method using the same organisms as for the plate assays was much more satisfactory, and was carried out as follows:

Miniature test-tubes, 5–6 cm. long, were made from glass tubing 3.95 ± 0.05 mm. internal diameter, and were closed with glass caps made from wider tubing. After being dry sterilized they were placed in racks and filled with inoculated melted nutrient agar. A fixed volume (usually 8.7 μl.) of fluid to be assayed was layered on top of the agar, the tubes were incubated at 37° C. overnight, and the length of the zone of inhibition was then measured. With 8.7 μl. of solution and with *S. typhi* as test organism 10 μg./ml. of N or 9 μg./ml. of penicillin could be detected, while with *Staph. aureus* (a more sensitive test organism for penicillin) about 0.3 μg./ml. of penicillin was measurable. With *S. typhi* the length of the zone of inhibition could be measured to within 0.1 mm., but with *Staph. aureus* the growth boundary was less sharp, though still serviceable.

One drawback of the vertical diffusion method, especially with narrow tubes, is that the test solution is liable to leak between the solidified medium and the walls of the tube, thus giving spuriously high results. This possibility was eliminated by making the density of the medium, and thus of any fluid exuding from it, greater than that of the test solution by incorporating sucrose to a concentration of 10%, which apparently did not interfere with the growth of either test organism. Davis *et al.* (1950) reported, apropos a similar diffusion method with capillary tubes open at both ends, that if the glass were made water-repellent the solid medium was much less readily dislodged; such treatment of the glass tubes may well overcome the risk of test solution creeping between tube and solidified medium, but it was not tested, as the sucrose medium gave very satisfactory results.

The medium was made as follows: To a series of capped 1-oz. phials containing 100 mg. of agar powder and 10 ml. tryptic meat digest broth were added while warm 10 ml. of a solution containing 20% (w/v) sucrose and 2% glucose, sterilized by Seitz-filtration. Before use 2–4 drops of a broth culture of the test organism were added to the melted contents of a phial which were filled into the tubes with a pointed Pasteur pipette.

Blood samples were collected from rats after snipping the tip of the tail which, with tne rest of the animal, was kept warm; the blood was taken into sterile capillaries which were sealed at both ends and placed in the refrigerator if not dealt with within one and a half hours. After centrifuging, the tube was cut just above the serum meniscus and the appropriate volume of serum, diluted if necessary with sterile normal rat serum, was transferred to the top of the seeded medium in an assay tube. Standards were set up at the same time with known amounts of antibiotic in the same volume of normal serum, and a standard curve was constructed from which the concentration of antibiotic in the unknowns could be read off. Two points should be mentioned:

(1) The curve was relatively steeper with just measurable amounts of antibiotic, possibly because oxygen is more available at the top of the tube. At the lowest level producing any effect, a few colonies appeared just below the surface of the agar, suggesting that better aeration had allowed them to develop although the concentration of antibiotic was slightly greater than that which inhibited the growth of the organisms just below them.

(2) When serum was assayed, a normal response was obtained with moderate or high concentrations of antibiotic. With low concentrations, however, the top 4–5 mm. of medium contained opaque material, usually in three distinct bands. This is presumably due to serum proteins which diffuse into the medium and are coagulated by acid generated by the bacteria in the lower parts of the tube. This opaque material obscures the position of the edge of the inhibition zone, but becomes transparent after half an hour or so if a drop of dilute alkali is added; if a little Indian ink is added to the alkali the upper boundary of the solid medium is more easily seen.

Cerebrospinal fluid was obtained by cisternal puncture of ether-anaesthetized rabbits, using the technique described by Markham *et al.* (1951). As normal cerebrospinal fluid free from antibiotic was not available, dilutions of the samples for assay and of standards were made in buffer.

Urine was obtained from female rats, catheterized with polythene tubing about 1 mm. in diameter, and was collected in tared phials and stored in the refrigerator.

RESULTS

Antibacterial Action

Potency and Range of Bacteria Inhibited.—The results of dilution tests in fluid medium with twofold dilution steps, carried out in parallel with N and penicillin, are summarized in Table I. Those for N broadly agree with the results of semi-quantitative streaking tests on solid medium done by Miss P. F. Boyd (unpublished). With both antibiotics the titre at 48 hours was either the same as that at 24 hours or was not more than one tube lower.

TABLE I

COMPARATIVE ANTIBACTERIAL TITRES OF CEPHALOSPORIN N AND PENICILLIN G

Organism	Inoculum. Broth Culture Diluted 1 in	Medium	Lowest Concn. (μg./ml.) Preventing Growth for 24 hr.	
			N	Penicillin
Staph. aureus NCTC 6571	1,000	HB	(*a*) 20 (*b*) 10	0·038 <0·095
Staph. aureus (penicillin resistant) ..	1,000	HB	1,000	13
M. tetragenus NCTC 7501	1,000	HB	10	<0·095
Sarcina lutea NCTC 248	1,000	HB	10	<0·095
Strep. pyogenes CN 10	10	HB+10% S	5	<0·095
Strep. viridans NCTC 3165	10	HB+10% S	10	0·19
Pneumococcus type I CN 33	10	HB+10% S	10	0·19
S. typhi avirulent ..	1,000	HB	(*a*) 10 (*b*) 5	4·8 6·0
S. typhi virulent ..	1,000	HB	(*a*) 5 (*b*) 6	4·8 >6·0
S. paratyphi B ..	1,000	HB	(*a*) 10 (*b*) 5	9·6 >6·0
S. paratyphi C ..	1,000	HB	(*a*) 5 (*b*) 5	0·3 0·75
S. typhimurium ..	1,000	HB	(*a*) 5 (*b*) 5	9·6 >6·0
S. enteritidis ..	1,000	HB	(*a*) 5 (*b*) 5	9·6 >6·0
Bact. friedländeri NCTC 5054 ..	1,000 100	HB HB+10% S	20 10	19·2 >6·0
Sh. shigae *Sh. sonnei* *Sh. flexneri* Z *Bact. coli*, type I	1,000	HB	>40	>6·0
Proteus vulgaris NCTC 3811 ..	1,000	HB	20	>6·0
Vibrio cholerae ..	100	HB	10	6·0
B. subtilis NCTC 1379	100	HB	5	0·19
B. anthracis avirulent	1,000	HB	5	<0·095
C. xerosis NCTC 7243	10	HB+10% S	20	0·38
C. diphtheriae gravis	10	HB+10% S	5	0·19
C. diphtheriae intermedius	10	HB+10% S	20	<0·095
C. diphtheriae mitis ..	10	HB+10% S	20	0·38
C. ovis	10	HB+10% S	10	0·38
L. monocytogenes NCTC 2167 ..	Undil.	Dextrose-phosphate	5	0·19
Br. abortus	,,	Liver broth	5	>6·0
P. pestis NCTC 144 ..	,,	HB+10% S	5	1·5
P. lepiseptica ..	,,	HB+10% S	10	1·5
P. muriseptica NCTC 948	,,	HB+10% S	10	3·0
N. meningitidis ..	,,	HB+20% S	5	<0·095
N. gonorrhoeae NCTC 8375	,,	HB+20% S	5	0·19
H. pertussis CN 134	,,	HB+20% S	5	0·75
H. influenzae (incubated 4 days) ..	,,	Fildes'	40	6·0
Cl. welchii NCTC 6125	100	HB+5% S	5	0·19
Cl. sporogenes NCTC 532	100	HB+5% S	5	0·19
Cl. tetani NCTC 279	100	HB+5% S	5	<0·095
Cl. septique NCTC 547	100	HB+5% S	5	0·19
Myco. tuberculosis H37Rv (human) (incubated 6 days)	*	Dubos	>40	>6·0
Myco. tuberculosis Branch (bovine) (incubated 6 days)	*	Dubos	>40	>6·0
Actinomyces graminis (Bostroem) ..	10	HB+10% S	>40	6·0

The inoculum was 0·02 ml. of a broth culture of the organism, diluted as indicated, per 2 ml. of medium.
HB = Ox heart infusion broth.
S = Rabbit serum, in percentage indicated.
* For *Myco. tuberculosis* the inoculum was 0·1 ml. of an undiluted 6-day culture in Dubos medium, per 5 ml.

The majority of the organisms were inhibited by 5–20 μg. of N/ml.; none of those tested were more sensitive than this, and a few were not affected by 40 μg./ml., the highest concentration used.

Nature of Antibacterial Action.—Viable counts showed that the action of N on *Staph. aureus* NCTC 6571 in tryptic meat digest broth was bactericidal, the count progressively falling to zero within 24 hours or so of the start of incubation. The rate of killing was the same with a just inhibitory concentration as with three times this concentration.

Combined Action of N and Cephalosporin P_1.—Since these two antibiotics are formed concurrently by the same cephalosporium (Burton and Abraham, 1951 ; Crawford *et al.*, 1952), it was of interest to study their combined action.

In the first experiment, with *Staph. aureus* NCTC 6571, tubes of tryptic meat digest broth were made up containing 2.25, 0.75, and 0.25 μg./ml. of crystalline cephalosporin P_1 (abbreviated here to "P"). These concentrations are above, approximately at, and below the minimum inhibitory concentration. A similar set of tubes contained in addition about one-fifth the minimum inhibitory concentration of N. Six more tubes contained three concentrations of N, with and without one-fifth the minimum inhibitory concentration of P. The warmed tubes were inoculated with approximately equal numbers of staphylococci and pour-plate viable counts were made at intervals.

The results were quite clear-cut and showed that the presence of a low concentration of P or N had a slightly antagonistic effect on the antibacterial action of the other.

An attempt was made to repeat the experiment with an avirulent strain of *S. typhi.* However, as the highest concentration of P that was used (0.1 mg./ml.) had no effect on the growth of the organism, the fact that it did not influence the killing effect of N may have little significance.

Cross-resistance.—Staph. aureus NCTC 6571 made more resistant to penicillin is more resistant to N, and vice versa.

Toxicity

Intravenous Injection (Mice).—The slow injection of as much as 100 mg. of N in 0.45 ml. distilled water into the tail vein in nine 20-g. mice failed to produce the slightest noticeable effect in seven of them, either immediately or during the subsequent seven days. Of the other two mice one showed slight waltzing movements and the other appeared slightly sick, but these effects lasted a few minutes only.

By contrast, mice were killed by lower doses of penicillin given in the same way and in the same strength (w/v) solution in distilled water (Table II).

TABLE II
TOXICITY OF CRYSTALLINE PENICILLIN G GIVEN INTRAVENOUSLY TO 20-G. MICE

Dose of Penicillin (mg.)	Mice Injected	Died	Survived
90	4	4	0
70	4	3	1
60	4	1	3
50	5	1	4

All the mice which died did so within 4–60 minutes of the injection, except one receiving 70 mg., which survived between 2½ and 15 hours. The solution, which was injected slowly into the tail vein, contained 20% (w/v) of penicillin in distilled water.

The toxicity of penicillin is said to be related to that of the cation, and it is quite possible that the three samples of N used here, having 32, 29, and 29 units/mg., contained less total cation than did the two batches of penicillin.

Intracisternal Injection (Rabbits).—Ten mg. of N in 0.5 ml. distilled water administered intracisternally to a 3 kg. rabbit caused mild disorientation and muscular weakness for several hours, but the animal appeared almost normal on the following day. Samples of cerebrospinal fluid withdrawn after 5 hr. 20 min. and after 23 hr. contained 0.24 and <0.012 mg./ml., the lowest concentration of N detectable, respectively. Both samples formed a tenuous clot and were cloudy from the presence of cells, mainly polymorphonuclear leucocytes with a few lymphocytes.

Another rabbit, weighing 2.8 kg., received 55 mg. N in 0.52 ml. water. As soon as the effect of the ether had worn off, the animal showed twitching and weakness, and made unsuccessful attempts to stand. After about an hour it became drowsy and nystagmus was evident. Three hours after the injection the animal was comatose, but thereafter it slowly recovered, though even after 24 hours it was weak. Samples of cerebrospinal fluid taken after 5 hr. 20 min. and after 24 hr. contained 1.8 and 0.065 mg. of N/ml. respectively. Both samples were cloudy.

Penicillin was injected into two rabbits of 2.7 kg. in the same way ; 5 mg. in 0.5 ml. of distilled water or normal saline caused fits leading to death after about 45 and 30 minutes respectively.

The rate of disappearance of N from the cerebrospinal fluid would appear to be of the same order as that reported for penicillin.

Absorption, Distribution in the Body, and Excretion

Distribution of N in the Blood.—Experiments in which N was added to heparinized rabbit blood, and then estimated in the plasma separated therefrom at intervals, showed that, like penicillin, N does not appreciably enter the red blood corpuscles.

Disappearance from Serum after Intravenous Injection.—The antibiotics, dissolved in 0.5–1.5 ml. of very dilute phosphate buffer or normal saline, were injected into the exposed saphenous vein of anaesthetized rats. Blood samples were taken at intervals, and the antibiotic content of the serum was plotted against time after injection. From the smoothed curves, serum levels of antibiotic at 10, 20, 30, 40, 60, 80, 100, 120, 150, and 180 minutes after injection were read off and replotted as a percentage of the theoretical serum level at zero time (plotted logarithmically), against time after injection. The results are shown in Fig. 1.

The theoretical level at zero time was calculated on the assumptions that the blood volume is 1/13th of the body weight, and that the volume of corpuscles is 40% of the volume of blood.

Concentration in Serum and Excretion After Subcutaneous Injection.—The results of two typical experiments in rats are shown in Fig. 2. The results of all the experiments are summarized in Fig. 3 (serum levels) and Fig. 4 (excretion data). As might be expected, the rate of excretion of both antibiotics was closely dependent on their concentration in the serum, but was not influenced by the rate of urine formation.

The general pattern of excretion was similar for the two substances, though less N was recovered than penicillin, and its peak rate of excretion occurred slightly later.

Concentration in Serum and Excretion after Gastro-intestinal Administration.—Three mice received 25 mg. of N by stomach tube ; three other mice received 7.5 mg. of penicillin by the same route. Four hours later the animals were killed, and the amounts of antibiotic remaining in the gut and present in the urine were measured. In all the mice well over 90% of the dose could not be accounted for, presumably partly because of the destruction by acid gastric juice. Of the residual material, less penicillin than N was found in the gut and more in the urine.

To avoid destruction by acid in the stomach, female rats were anaesthetized with ether, catheterized, and 1 ml. of solution of antibiotic in water was introduced through an abdominal incision into the duodenum. The abdominal cavity was closed, and urine and blood samples were collected at intervals. After about 4 hours the rat was killed and the contents of the small intestine were washed out with buffer and assayed. These results and the urine assays are summarized in Table III ; with regard to serum levels, that of penicillin rose to a low but detectable level after 30–60 min. and persisted for two hours before gradually falling. The peak level was from one-tenth to one-thirtieth of that after a comparable dose of penicillin given subcutaneously. N could not be detected in the serum at any time, because of the much lower sensitivity of test organisms to this antibiotic. Table III confirms the impression gained from the mouse experiments that N is less readily absorbed from the intestine than is penicillin.

Although distilled water was used for dissolving the antibiotics for this experiment the solutions probably approached the tonicity of normal saline. At any rate, pieces of duodenum taken at the end

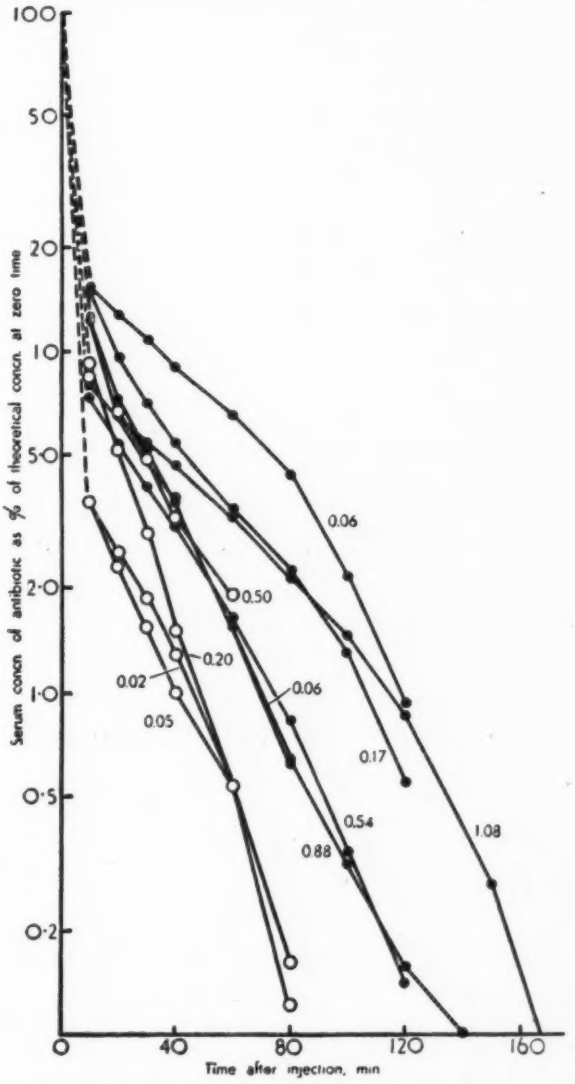

FIG. 1.—Concentration in serum of cephalosporin N (solid circles) and penicillin G (open circles) at different times after intravenous injection into rats of the doses (g./kg. body weight) indicated. The concentration is expressed on a logarithmic scale.

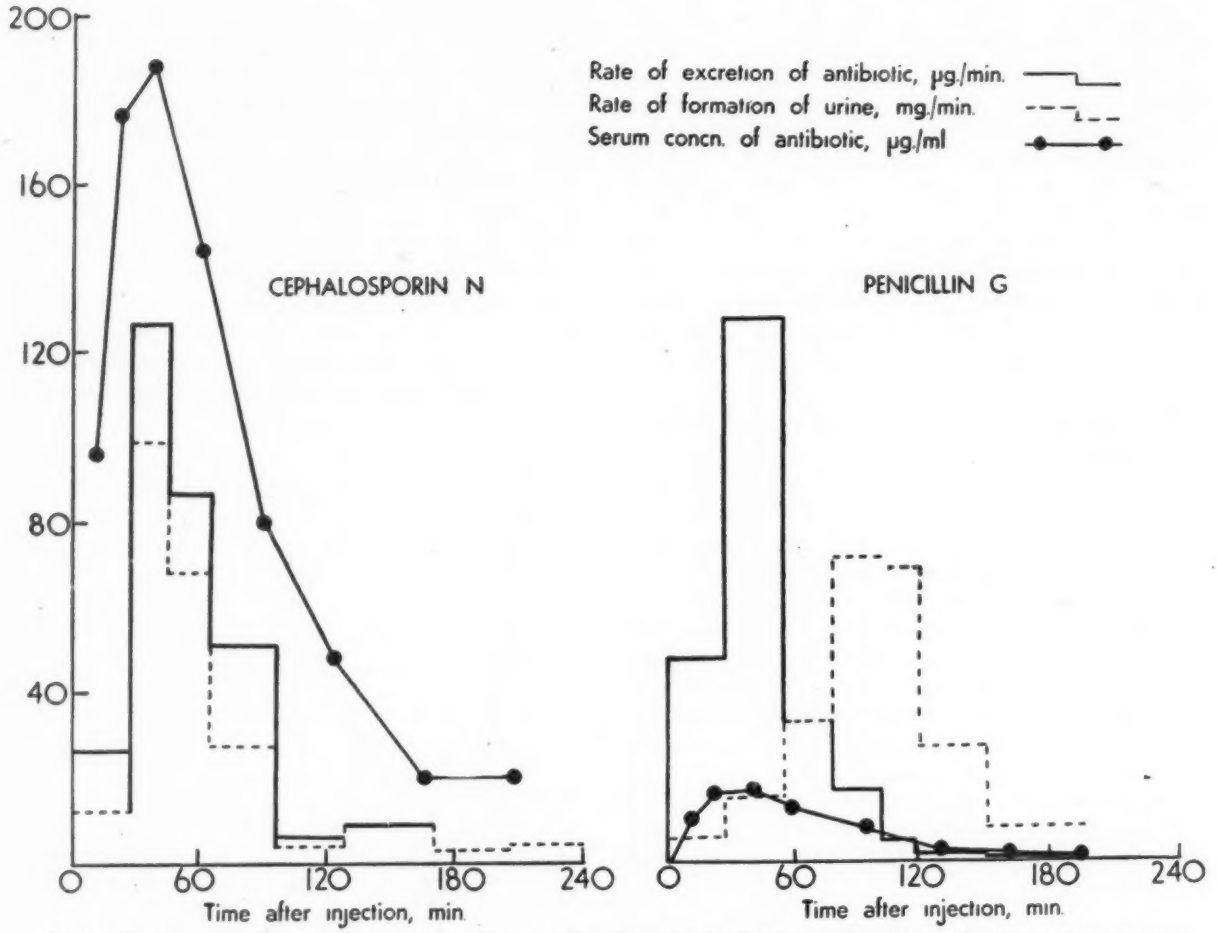

FIG. 2.—Typical results of subcutaneous injection of 30 mg. cephalosporin N (left) and 10 mg. penicillin G (right) into 200-g. rats. In the former the rate of excretion of N happens to coincide with the rate of formation of urine, but usually there is no correlation, as on the right.

of some of the experiments showed histologically no signs of irritation or of damage to the villi.

TABLE III

DISTRIBUTION OF PENICILLIN OR N AFTER DIRECT INTRODUCTION IN 1 ML. WATER INTO THE DUODENUM OF THE RAT

Dose (g./kg.)	Per Cent of Dose Recovered	
	In Urine	From Gut
Penicillin:		
0·06	11·1	13·8
0·11	8·0	33
0·15	20·7	11·5
0·23	11·1	25
0·26	4·1	24
Cephalosporin N:		
0·17	<2·3	41·3
0·30	Lost	30
0·38	<0·15	52
0·55	<0·5	38
0·86	<0·1	32

Experiments lasted about 4 hr. Rats weighed 165–200 g. Urine was collected in several fractions by catheter.

Effect of Animal Passage on Hydrophilic Character of N.—It seemed possible that N might be changed during its passage through the animal body. No information on this point was obtained other than that the active material in the urine of rats which had received N intravenously was, like N itself (but unlike penicillin G), not extracted preferentially into butyl acetate from watery solution at *p*H 2.

DISCUSSION

The investigations of Abraham *et al.* (1953) leave no doubt that cephalosporin N is a form of penicillin with physical and chemical properties distinguishing it sharply from the types of penicillin known hitherto. The experiments described in this paper were undertaken to see if these different properties conferred on N a distinctive behaviour in the animal body and towards bacteria. In

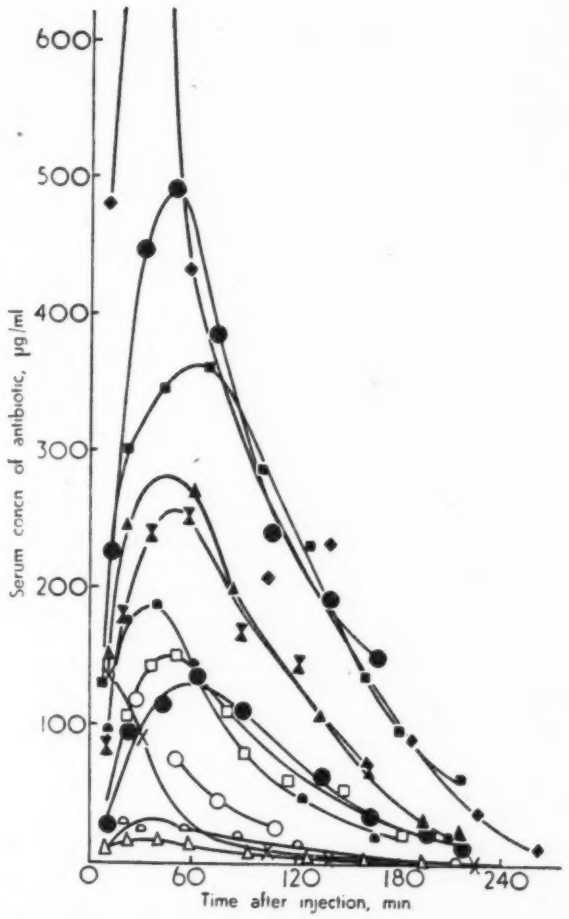

FIG. 3.—Serum concentration of cephalosporin N (solid points) and penicillin G (open points) at different times after subcutaneous injection of various doses into rats. Doses (g./kg. body weight) were as follows: Cephalosporin N: 0.54, ◆——◆, ●——● (upper); 0.49, ■——■, ▲——▲; 0.33, ⧗——⧗; 0.23, ●——● (lower); 0.15, ◓——◓. Penicillin G: 0.45, □——□; 0.15, ×——×; ○——○; ⌂——⌂; 0.05, △——△.

particular an answer was sought as to whether it might have therapeutic advantages over penicillin G and similar penicillins.

Impure N is certainly as little toxic to mice as "pure" penicillin G, possibly less so.

Against most bacteria—with certain exceptions important clinically, e.g., *Myco tuberculosis, Salmonella* spp., penicillin-resistant staphylococci, *Bact. friedländeri,* etc., against which it is less active—N seems to have a uniform minimum inhibitory concentration of the order of 10 µg./ml. Penicillin, while having a similar potency against several Gram negative organisms, including *S. typhi,* is over a hundred times more active against many Gram positive pathogens. Thus, in the most general terms it is only against certain organisms causing diseases not normally treated by penicillin that the potency of the two antibiotics is comparable, weight for weight.

In the experiments on absorption and excretion there was a wide variation of response from animal to animal with both drugs which could not be related to sex, body weight, rate of urine formation, or any other factor, but the following statements can be made with some certainty:

1. After intravenous administration N persists longer and gives a higher level in the serum at any one time than does an approximately equimolar dose of penicillin. Initially both drugs rapidly leave the circulation, more than 80% of the dose having disappeared during the first 10 minutes; thereafter the fall in the serum concentration of N tends to be more gradual than that of penicillin, though the relative rate of excretion of both increases slightly towards the end of the experiment. The slight concavity at the beginning of the N curves (Fig. 1) suggests that the initial escape of N from the blood stream is not as rapid as that of penicillin.

2. After subcutaneous administration the serum level of N is again higher than that of penicillin (Fig. 3), the peak levels occurring after 30–60 and 15–45 minutes respectively. Urinary excretion is substantially complete with both drugs after 100–150 minutes, and the peak occurs at 30–40 minutes.

3. For a given serum concentration penicillin is more rapidly excreted in the urine than is N. However, the latter persists in the serum only slightly longer than does penicillin, presumably due to its greater destruction or disappearance in the animal body, as shown by its lower total urinary recovery.

4. Though quantitative experiments on absorption from the gut were incomplete, owing to the concentration of N in the various body fluids being scarcely if at all detectable, it is clear that N is absorbed from the intestine more slowly than is penicillin.

The doses of penicillin given in the animal experiments were, from a therapeutic point of view, very large. This arose from the conviction that the substances should be compared on a roughly equimolar basis. Large amounts of N, and therefore of penicillin also, had to be used in order that readily measurable amounts of N might be present in the body fluids. (The molecular weight of N is not known. In this work it was assumed to be twice or three times that of penicillin G.)

Because the differences in behaviour between penicillin G and N in the animal body are not striking, and the advantages in antibacterial action

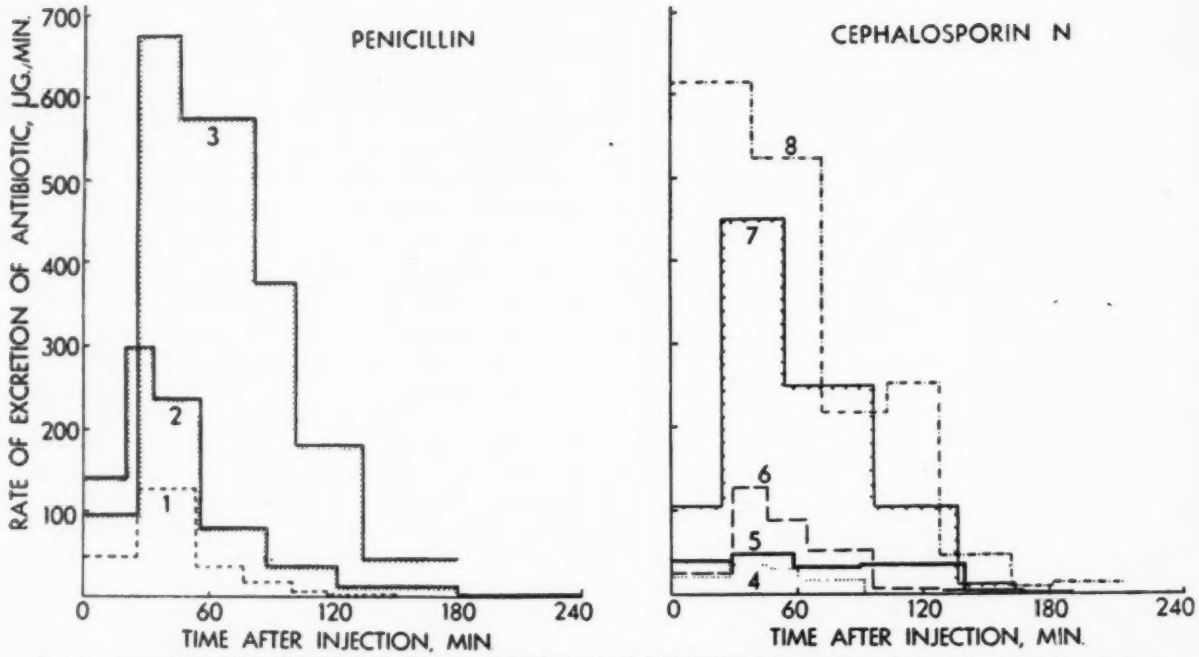

FIG. 4.—Rate of excretion of penicillin G (left) and cephalosporin N (right) after subcutaneous injection of various doses in rats. Dosages (g./kg. body weight) and per cent of dose recovered in urine (in brackets) were as follows:—Penicillin: Exp. 1, 0.05 (61%); Exp. 2, 0.15 (57%); Exp. 3, 0.45 (58%). Cephalosporin N: Exp. 4, 0.30 (4%); Exp. 5, 0.23 (12.7%); Exp. 6, 0.15 (23%); Exp. 7, 0.49 (35%); Exp. 8, 0.49 (63%). In Exp. 8 the high recovery and the earliness of the peak of excretion suggest that inadvertently some of the dose may have been given intravenously.

are either nil or more or less strongly in favour of penicillin (depending on the organism under consideration), cephalosporin N would not appear to be so good a therapeutic agent as penicillin G, in spite of the possible greater toxicity of the latter, except perhaps in one respect. Now that there is considerable fear of using chloromycetin because of its effect on the bone marrow, it might be worth exploring anew the possibility of using penicillin for the treatment of typhoid fever. Since N appears to be less readily absorbed from the gut than penicillin, it could conceivably be more effective than the latter if it could be brought into the lower small intestine. Unfortunately N, like penicillin, is subject to destruction in the stomach by the acid gastric juice, and in the intestine by penicillinase-producing bacteria.

On present knowledge it seems safe to conclude that N has no immediate therapeutic future.

SUMMARY

1. The minimum inhibitory concentrations of cephalosporin N and penicillin G against a number of micro-organisms have been measured.

2. The two substances have been compared in respect of their absorption from subcutaneous tissue and intestine, the maintenance of serum levels, and excretion by the kidneys. While their general behaviour is the same, N appears to be absorbed more slowly from the intestine, and to be excreted at a lower rate and in smaller amount than penicillin.

3. The toxicity of impure N, given intravenously to mice or intracisternally to rabbits, is less than that of penicillin G.

We are greatly indebted to Mr. N. Smith, who carried out all the antibacterial titrations and performed the viable counts; to Dr. A. G. Sanders, who assisted with the intracisternal punctures; and to Mr. J. Kent and Miss E. J. Page, who helped with the animal and other work.

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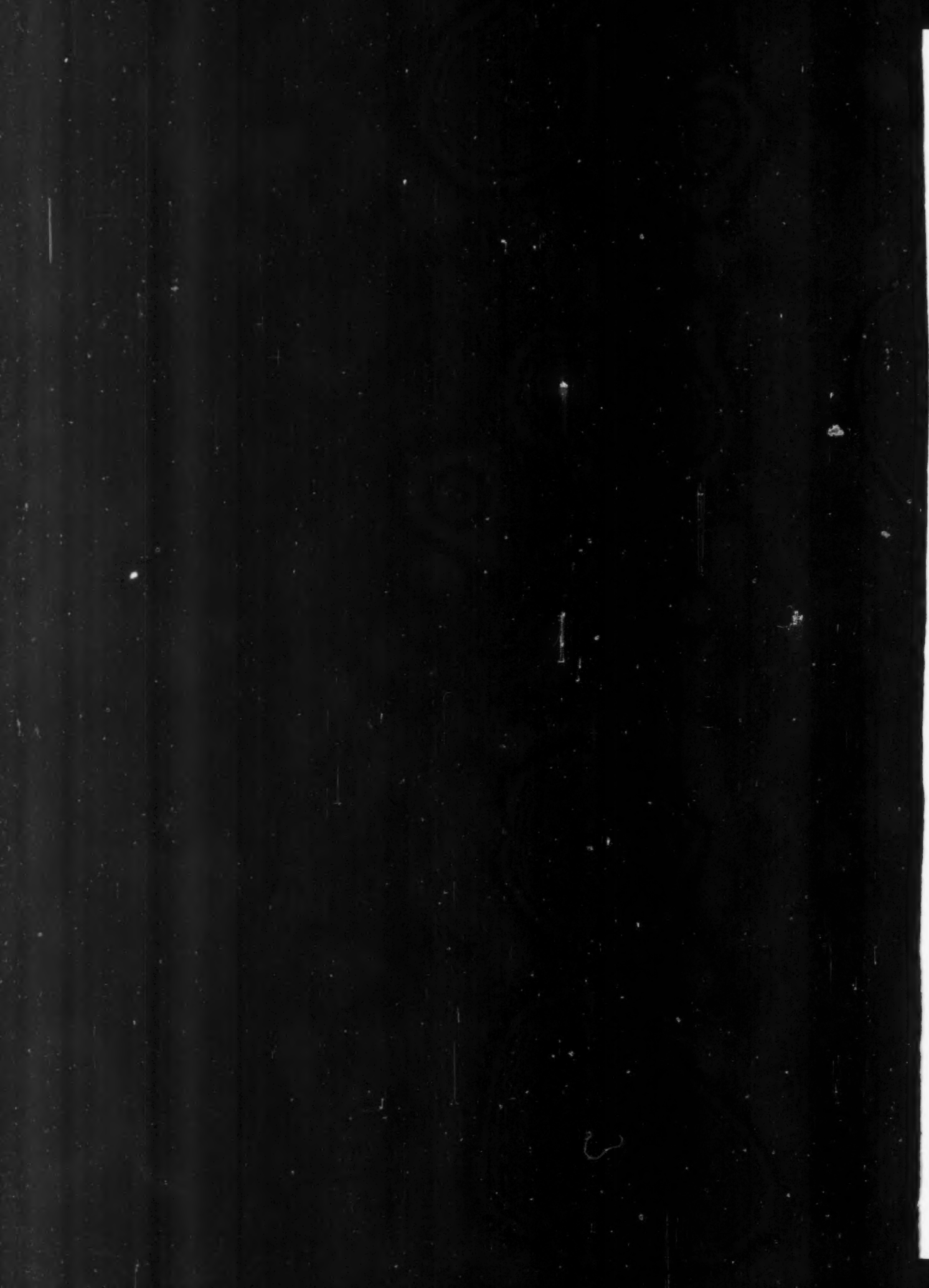

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